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(54) Title: IMPROVED METHODS FOR MAKING HORMONE HETERODIMERS

(57) Abstract

The present invention relates to a method for preparing heterodimeric analogs of cysteine knot proteins. More specifically, the invention relates to a method for forming a subunit combination of a cystein knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of (a) attaching a dimerization domain to the amino termini of both an α-subunit and a β-subunit of a cystein knot protein; and (b) dimerizing the α-subunit and a β-subunit to form a heterodimeric protein analog. In another embodiment, the invention relates to a method for forming a subunit combination of a cystein knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of (a) attaching a dimerization domain to the amino terminus of an α-subunit and the carboxy terminus of a β-subunit of a cystein knot protein; and (b) dimerizing the α-subunit and β-subunit to form a heterodimeric protein analog.

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IMPROVED METHOD FOR MAKING HORMONE HETERODIMERS

BACKGROUND OF THE INVENTION

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The experiments in this application were supported by the National Institutes of Health, Grant Number HD14907. The United States Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided by the terms of Grant Number HD14907 awarded by the National Institutes of Health.

Field of the Invention

The present invention relates to a method for preparing heterodimeric analogs of cysteine knot proteins. More specifically, the invention relates to a method for forming a subunit combination of a cysteine knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of (a) attaching a dimerization domain to the amino termini of both an α-subunit and a β-subunit of a cysteine knot protein; and (b) dimerizing the α-subunit and β-subunit to form a heterodimeric protein analog. In another embodiment, the invention relates to a method for forming a subunit combination of a cysteine knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of (a) attaching a dimerization domain to the amino terminus of an α-subunit and

the carboxy terminus of a β-subunit of a cysteine knot protein; and (b) dimerizing the α-subunit and β-subunit to form a heterodimeric protein analog.

Description of the Background

The disclosures referred to herein to illustrate the background of the invention and to provide additional detail with respect to its practice are incorporated herein by reference and, for convenience, are numerically referenced in the following text and respectively grouped in the appended bibliography.

The glycoprotein hormones and their biological actions

The glycoprotein hormone family (1-3) consists of three α, β heterodimeric glycoproteins found in the anterior pituitary gland where they are made and includes luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH). These hormones are found in most, if not all vertebrates. In some species, a glycoprotein hormone structurally similar to LH is found in the placenta wherein it is synthesized. The human placental hormone is known as human chorionic gonadotropin (hCG). In primates, significant quantities of all the hormones are also found as excretion products in urine. Urine from pregnant women serves as a convenient source of hCG. After menopause, when the secretion of LH and FSH from the anterior pituitary is greatly increased, significant quantities of LH and FSH are found in the urine and are termed human menopausal gonadotropins (hMG). Urine from menopausal women serves as an important source of LH and FSH activities. Urinary hormones (hCG, hMG, hFSH) and recombinant hormones have important clinical and commercial uses.

Gonadotropins such as CG, LH, and FSH play a major role in the reproductive process (4) while the structurally related hormone, TSH, is important for thyroid function. In women, FSH plays a crucial role in the development of follicles that can be ovulated, primarily through its influence on granulosa cells. LH synergizes with FSH and is normally essential for processes of ovulation, luteinization, and luteal function. Nonetheless, high LH levels can reduce fertility and are thought partly responsible for the loss of fertility associated with polycystic ovarian disease. hCG is important for maintenance of pregnancy and its early neutralization leads to infertility. In males LH is required for puberty and, in its absence, there is a failure to acquire the sexual attributes and fertility of an adult. The biological and clinical activities of these hormones are reviewed extensively in several textbooks including those by Yen and Jaffe (4), Adashi, Rock, and Rosenwaks (5), and DeGroot (6).

Both hCG and LH bind to luteinizing hormone receptors (LHR). In the testis, LHR are found primarily in the Leydig cells. In the ovary, LHR are found primarily in thecal cells, FSH-stimulated granulosa cells, and luteal cells. The major role of LH is to stimulate the formation of steroid hormones including the androgens testosterone and androstenedione (Leydig and thecal cells) and progesterone (FSH-stimulated granulosa, thecal, and luteal cells). LH also causes ovulation of mature follicles. While hCG is normally produced only by the placenta during pregnancy, due to its high affinity for LH receptors, the ease with which it can be purified from urine, and its long biological half-life, hCG has been widely used as a substitute for LH. Important clinical uses for hCG include stimulation of fertility in males and induction of ovulation in females.

FSH binds to FSH receptors (FSHR) located primarily in the Sertoli cells of the testis and the granulosa cells of the ovaries. The primary roles of FSH are to stimulate the conversion of androgens to estrogens, to promote the synthesis of inhibin and activin, to promote the development of Sertoli and granulosa cells, and to stimulate

gamete maturation. The effect of FSH on granulosa cells leads to follicular maturation, a process during which the oocyte is prepared for ovulation and in which the granulosa cells acquire the ability to respond to LH. Follicle maturation is essential for the ability of LH to induce ovulation.

The differences in the effects of FSH and LH and the complex endocrine interactions between the two hormones cause them to have synergistic effects. For example, normal estrogen production is due to the effect of LH on androgen formation and the influence of FSH on the conversion of androgens to estradiol. Estrogens can inhibit the secretion of FSH and potentiate the secretion of LH. The ability of androgens to be converted to estrogens in non-ovarian tissues can disrupt this complex feedback interaction between steroidogenesis and the secretion of FSH and LH. For this reason, the ratio of LH/FSH activity as well as the absolute hormone levels in blood are important for reproductive functions such as ovulation of the proper number of oocytes during the menstrual and estrus cycles. Other hormones including activin and inhibin can exert an influence on this process, primarily through their influence on FSH secretion from the pituitary gland and their influence on the ovarian response to FSH.

TSH is produced in the anterior pituitary gland and its major function is to regulate the activity of the thyroid gland, causing it to synthesize and release thyroxin. Circulating levels of TSH and thyroxin are usually regulated by a negative feedback mechanism. Increases in TSH secretion usually lead to increased thyroxin synthesis and secretion by the thyroid. As thyroxin levels increase, the secretion of TSH is decreased. In this way there is a balance between the level of TSH and thyroid hormone. High levels of TSH can also stimulate the thyroid gland to remove iodine from circulation. Clinically, TSH can be used to promote the uptake of radioactive iodine and death of the thyroid cells. This form of thyroidectomy has been used to remove hyperactive thyroid tissues.

Uses of glycoprotein hormones and the desirability of novel hormone analogs

Hormones with FSH and LH activities are routinely used in the treatment of human infertility, a problem experienced by approximately 10-15% of all couples (7,8). A major cause of female infertility is polycystic ovarian disease or syndrome, a condition in which endogenous LH levels often appear to be elevated. In principle, infertility caused by inappropriately high LH activity could be suppressed by administration of an inhibitory hormone analog that competed with LH for binding to LHR. It has been known for many years (9,10) that it is possible to prepare analogs of hCG that act as LH antagonists by removing all or part of the oligosaccharides from the hormone. While it is possible to remove most of the oligosaccharides using endonucleases or exonucleases, in practice, it is not practical to remove all of them without denaturing the hormones. The remaining sugars can serve as substrates for enzymes and other factors that can hasten removal of the proteins from circulation (11-13). One potential means of avoiding this problem is to prepare analogs that have been genetically deglycosylated (i.e., by replacing or deleting amino acids in the signals needed for N-linked glycosylation). These signals have the amino acid sequence Asn-Xaa-Ser/Thr where Asn is asparagine, Xaa is any amino acid except proline, and Ser/Thr are serine or threonine. To disrupt glycosylation, Asn can be changed to any other amino acid, Xaa can be changed to proline and/or Ser or Thr can be changed to any other amino acids.

Using genetic deglycosylation, it has been shown that the oligosaccharide from the hCG α-subunit at Asn52 has the greatest influence on signal transduction (10). Removal of this oligosaccharide leads to a substantial loss in hormone efficacy and enables the preparation of a partial agonist that can partially inhibit the response to hCG. However, because the other hormone oligosaccharides also influence signal transduction, preparation of the most potent antagonists requires that other N-linked

amino acids, particularly those on the α-subunit, be removed from the hormone (10). Unfortunately, removal of the α-subunit oligosaccharide at Asn52 reduces the abilities of the α- and β-subunits to combine (10,14-16). While small amounts of heterodimer do form and can be studied in a laboratory setting (10), preparation of larger quantities needed for potential therapeutic uses is impractical. Methods for improving the production of deglycosylated glycoprotein hormone analogs are desirable and, as described later, one such method involves addition of dimerization sequences to the hormone subunits.

Hormone analogs that have prolonged half-lives or universal activities also have potential important uses. It is well known that the half-lives of the subunits is significantly shorter than that of the heterodimers [reviewed in Moyle and Campbell (2)]. Because dimerization domains can potentiate formation of heterodimers, they can also reduce the rate of hormone dissociation and influence circulation time. Hormone analogs that serve as immunogens are also potentially important. Dimerization domains can contain high immunogenic amino acid sequences and therefore increase the immunogenicity of the analogs.

Structures of the glycoprotein hormones

The structures of the glycoprotein hormones have been studied for many years and the relative roles of the hormone subunits in receptor binding specificity are well-known (1). Glycoprotein hormones share a common α-subunit and differ in their hormone-specific β-subunits. The latter determine the biological and immunological properties of each hormone. Substitution of the α-subunit of any one hormone for that of another does not change the receptor binding properties of the new hormone. Substitution of the β-subunit is accompanied by a change in receptor binding specificity. Thus, when FSH β-subunit is substituted for the LH β-subunit, the recombined hormone acquires the properties of FSH and loses properties characteristic

of LH. The sequences of many hormone subunits were determined several years ago and have been confirmed by their genomic and cDNA sequences (17-21).

The crystal structure of hCG determined in two laboratories (22,23) showed that each subunit had the overall topology characteristic of cysteine knot proteins (24). Each subunit is divided into three large loops by disulfide bonds that create the cysteine knot. Since the relative positions of the cysteines in all the glycoprotein hormones are very similar, it is nearly certain that the β-subunits of LH, FSH, and TSH will also have a cysteine knot architecture. The β-subunit differs from the α-subunit in one important aspect, namely the presence of an additional sequence of approximately twenty amino acids that is attached to the C-terminal cysteine of the cysteine knot. In the β-subunits of hLH, hCG, hFSH, and hTSH, the seatbelt corresponds to amino acid residues Gly91-Cys110, Ala91-Cys110, Gly85-Cys104, and Gly86-Cys105, respectively. This sequence was termed the seatbelt (22) because it is wrapped around the α-subunit and forms a disulfide bond with a cysteine in β-subunit loop 1 to stabilize the heterodimer. As reviewed by Ruddon and colleagues, the cysteine knot that latches the seatbelt appears to be one of the final steps in β-subunit folding and appears to occur after the two subunits have combined (25).

Several attempts have been made to identify portions of the α- and β-subunits of the hormones that are responsible for their unique biological properties. Earlier studies were based on chemical modifications of the hormones (1). Modifications were described that reduced the biological activities of the hormones. Due to the complexity of the hormones, this approach was usually unable to identify single amino acid residues that were involved in receptor binding or binding specificity. In an attempt to simplify the problem of identifying residues involved in receptor binding, some investigators prepared synthetic peptides corresponding to partial sequences of the α- and β-subunits and monitored their abilities to inhibit binding of ^{125}I-hCG and ^{125}I-hFSH to LH and FSH receptors. Synthetic peptides corresponding to amino acid residues of

hCG β-subunit 38-57 or hFSH β-subunit 31-52 appear to have higher abilities than most other peptides in these assays (26-29). However, they have extremely low affinities for the receptors, an observation that precludes their practical use.

A breakthrough in the ability to make and characterize glycoprotein hormone analogs came in 1985 when genetically engineered mammalian cells were first shown to express biologically active hCG heterodimers (30). Since that time several laboratories have used mammalian cells to express glycoprotein hormone analogs that are capable of binding to receptors and inducing or inhibiting signal transduction (14,31-37). These analogs appear to be glycosylated similarly to the naturally occurring hormones. In these procedures one introduces a "gene" that encodes the desired amino acid sequences into mammalian cells downstream of a promoter. Construction of these genes is a standard recombinant DNA procedure. By changing, adding and/or deleting codons in the hormone α- or β-subunit cDNAs or genomic DNA fragments using standard procedures, it is possible to build gene constructs that encode the desired analogs (38,39). When these constructs are transfected into mammalian cells by calcium phosphate precipitation, electroporation, or other standard protocols (38-40), they direct the synthesis of the hormone analogs and their secretion into the culture media. These media can be assayed for the presence of immunological or biological activity (31,32,41). Unfortunately, not all such constructs yield practical amounts of heterodimers. This is especially evident with hormones that lack one or more glycosylation signals.

Using mammalian cell expression systems to make hormone analogs, Campbell, et al. (31) showed that it was possible to switch the receptor binding activity of hCG. They engineered an analog that was chemically and immunologically more similar to hCG than hFSH, but that bound to FSH receptors much better than hCG and had only slightly higher affinity for LH receptors than FSH. Subsequent reports (33) showed that it was possible to prepare analogs of hCG that had a high affinity for both LH and

FSH receptors. This was accomplished by replacing hCG seatbelt residues 101-109 with their hFSH β-subunit counterparts (i.e., hFSH β-subunit residues 95-103). These hCG analogs (31,33) elicit signal transduction at either LH and/or FSH receptors. This demonstrated that the seatbelt of the β-subunit had a major influence on receptor binding specificity. It is anticipated that removing the oligosaccharides from analogs in which the specificity is modified by substitutions in the seatbelt will reduce their efficacy and cause them to become partial agonists and/or antagonists. Their ability to bind to receptors requires that the β-subunits of these analogs combine with the α-subunit to form heterodimers. The method described here will be useful for expressing these analogs as heterodimers and represents a significant advance in heterodimer preparation.

Slaughter et al. (42) showed that an interaction between the N-terminal portion of hCG β-subunit and the seatbelt had a substantial influence on subunit combination. Removal of the hCG β-subunit N-terminus led to loss in its ability to combine with the α-subunit to form a heterodimer. This could be restored in part by changing the seatbelt residues of the β-subunit to those found in the β-subunit of hFSH. This suggested that interactions between different parts of the hormone subunits had significant roles in subunit combination. It also suggested that subunit combination was complex and that any modification of this region of the hCG β-subunit might be expected to interfere with subunit combination. Indeed, work by Keutmann and colleagues (43) showed that synthetic peptides similar in structure to the N-Terminal region of the hCG β-subunit inhibited subunit combination and that this portion of hCG was likely to be near the α-subunit.

Sugahara et al. (44) showed that a fusion protein between the α- and β-subunits would lead to a protein that had many of the same properties as the heterodimeric parental molecule, including the ability to bind to receptors. Nonetheless, these

analogs have all the amino acids of the protein connected together in a single-chain and therefore differ substantially from proteins that have two subunits. Unlike single chain proteins that are folded differently from the native hormones, hormone analogs that have two separate subunits similar to those found naturally would be expected to have receptor binding and immunological properties that are more similar to those of the parental molecules.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the coding sequence of the hCG β-subunit cDNA between the XhoI and BamHI sites except that the codons for amino acids 2-8 have been deleted.

Figure 2 illustrates the coding sequence of a vector that encodes the hCG β-subunit signal sequence upstream (5') of bases that encode serine, cysteine, two glycines, and portions of the Fos dimerization domain (one letter code).

Figure 3 illustrates the coding sequence of a vector that encodes the hCG β-subunit signal sequence upstream (5') of bases that encode serine, cysteine, two glycines, and portions of the Jun dimerization domain (one letter code).

Figure 4 illustrates the sequences of oligonucleotides used in this work including primers used to create a cassette that permitted insertion of the Fos and Jun dimerization domain amino acid coding sequences into the construct illustrated in Figure 1.

Figure 5 shows the coding sequence of the entire Fos-hCG-α-subunit construct.

Figure 6 shows the coding sequence of the entire Jun-hCGβ'-subunit construct.

Figure 7 shows that the heterodimer containing the Fos-Jun amino acid sequences at its N-termini can stimulate signal transduction in a similar fashion as hCG.

Figure 8 shows that the heterodimer containing the Fos-Jun amino acid sequences at its N-termini can inhibit the binding of radioiodinated hCG to CHO cells expressing rat LH receptors in a similar fashion as hCG.

Figure 9 illustrates the sequence of an α-subunit construct that is lacking the glycosylation signal normally found at Asn52 of the mature human α-subunit.

Figure 10 illustrates the sequence of the Fos-α-subunit construct that is lacking the glycosylation signal corresponding to human α-subunit residue 52 caused by substitution of an aspartic acid residue for the asparagine normally found at this residue of the human α-subunit.

Figure 11 illustrates the sequence of the Fos-α-subunit construct in which the cysteine normally found at position 7 of the human α-subunit has been replaced by an alanine.

Figure 12 illustrates the sequence of the Jun-hCGβ'-subunit in which the tyrosine normally found in the hCG β-subunit at residue 37 has been replaced by a cysteine.

Figure 13 illustrates the Jun-hCGβ'-Y37C-C26A construct.

Figure 14 illustrates the Jun-hCGβ'-Y37C-C26A-δ92 construct.

Figure 15 illustrates the abilities of hFSH, hCG, Fos-Jun-hCG-SS/δseatbelt, and Fos-Jun-hCG-SS to stimulate signal transduction in CHO cells expressing human FSH receptors.

Figure 16 illustrates the abilities of bovine TSH, hCG, Fos-Jun-hCG-SS/δseatbelt, and Fos-Jun-hCG-SS to stimulate signal transduction in CHO cells expressing human FSH receptors.

Figure 17 illustrates the sequences of constructs of Fos-hCG α-subunit containing a furin cleavage site and the sequences of Jun-hCG β-subunit, Jun-hLH β-subunit, Jun-hFSH β-subunit, Jun-hTSH β-subunit, Jun-hCG/hFSH β-subunit chimera, and Jun-hCG/hTSH β-subunit chimera containing a furin cleavage site.

Figure 18 illustrates the sequences of constructs containing immunoglobulin dimerization domains at their N-termini.

Figure 19 illustrates the sequences of constructs containing immunoglobulin dimerization domains at their N-termini.

Figure 20 illustrates the amino acid sequences of β-subunit constructs containing the dimerization domain from Jun at the carboxyterminus of their dimerization domains.

SUMMARY OF THE INVENTION

The present invention pertains to a method for forming a subunit combination of a cysteine knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of:

(a) attaching a dimerization domain to the amino termini of both an α-subunit and a β-subunit of a cysteine knot protein; and

(b) dimerizing the α-subunit and β-subunit to form a heterodimeric protein analog.

In a preferred embodiment, a Fos dimerization sequence domain is attached to the amino-terminus of the β-subunit and a Jun dimerization sequence domain is attached to the amino-terminus of the α-subunit. In another preferred embodiment, a Fos dimerization sequence domain is attached to the amino-terminus of the α-subunit and a Jun dimerization sequence domain is attached to the amino-terminus of the β-subunit. A glycine or serine residue may be inserted between the Fos or Jun dimerization sequence domain and a furin cleavage site to facilitate cleavage of the dimerization sequence domain from the heterodimer. The dimerization domain may also be a heavy or light chain of an immunoglobulin. The heterodimeric protein analog may be a glycoprotein hormone heterodimer selected from the group consisting of hCG, hLH, hFSH, hTSH, TGFβ, PDGF, NGF, Veg1, bone morphogenic proteins, activin, inhibin, and analogs thereof. The heterodimeric protein analog may also be selected from the group consisting of hCG/hFSH chimeras, hCG/hTSH chimeras, deglycosylated hormones, truncated glycoprotein hormones, mutant glycoprotein hormones, and glycoprotein hormones containing an hCG carboxyl terminus. Protease cleavage sites may be incorporated between additional N-terminal sequences and the α-subunit and the β-subunit of the cysteine knot protein to remove the dimerization

domains from the heterodimeric protein analog. The cysteine knot protein may be a glycoprotein hormone heterodimer having the oligosaccharide genetically removed from the α-subunit at Asn52. The cysteine knot protein may also be a glycoprotein hormone heterodimer lacking a seatbelt.

In another embodiment, the present invention pertains to a method for forming a subunit combination of a cysteine knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of:

(a) attaching a dimerization domain to the amino terminus of an α-subunit and the carboxy terminus of a β-subunit of a cysteine knot protein; and

(b) dimerizing the α-subunit and β-subunit to form a heterodimeric protein analog.

In a preferred embodiment, a Fos dimerization sequence domain is attached to the carboxy-terminus of the β-subunit and a Jun dimerization sequence domain is attached to the amino-terminus of the α-subunit. In another preferred embodiment, a Fos dimerization sequence domain is attached to the amino-terminus of the α-subunit and a Jun dimerization sequence domain is attached to the carboxy terminus of the β-subunit. A protease cleavage site may be inserted between the dimerization sequence domain and the α-subunit and a protease cleavage site may be inserted between the dimerization sequence domain and the β-subunit. Preferably, the protease cleavage site is furin. The dimerization domain may be a heavy or light chain of an immunoglobulin. The heterodimeric protein analog may be selected from the group consisting of hCG/hFSH chimeras, hCG/hTSH chimeras, deglycosylated hormones, truncated glycoprotein hormones, mutant glycoprotein hormones, and glycoprotein hormones containing an hCG carboxyl terminus. The cysteine knot protein may be a glycoprotein hormone heterodimer having the oligosaccharide genetically removed from the α-subunit at Asn52.

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DETAILED DESCRIPTION OF THE INVENTION

Human chorionic gonadotropin (hCG), luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) are members of the heterodimeric glycoprotein hormone family. LH and hCG bind to LH receptors (LHR), FSH binds to FSH receptors (FSHR), and TSH binds to TSH receptors (TSHR). Interactions of LH and FSH with gonadal LHR and FSHR are essential for fertility. Interactions of TSH with TSHR are essential for proper functioning of the thyroid gland. All four hormones are heterodimers containing an α-subunit produced from the same gene and a hormone-specific β-subunit. The crystal structure of hCG shows that both subunits are members of the cysteine knot family of proteins. Because the endocrine activities of the heterodimers exceed those of the isolated subunits, it is usually desirable to prepare heterodimers in which the α- and β-subunits are combined similarly to the native hormones. Formation of non-native or mutant heterodimers is often impeded by changes to one or both subunits needed to elicit desirable properties. The present method promotes subunit combination of the cysteine knot family of proteins and thereby improves the synthesis of heterodimeric hormone analogs. This method involves attaching dimerization domains to the amino termini of both hormone subunits, a process that facilitates combination of the remainder of the proteins into active heterodimers. By incorporating appropriate protease cleavage sites between the additional N-terminal sequences and the α- and β-subunits, it is also possible to remove the dimerization domains from the hormone dimers to create hormone analogs that are similar in structure to the native molecules.

By employing the method of the present invention, it is possible to increase the efficiency of subunit combination for subunits that would otherwise not combine or that would combine very poorly by adding sequences dimerization domains to the N-terminus of each subunit. This method can also be applied to the preparation of

glycoprotein hormone heterodimers in which the oligosaccharide has been genetically removed from the α-subunit at Asn52. This method can also be used to produce heterodimers lacking the seatbelt. The method outlined in this invention should be useful for facilitating the dimerization of any cysteine knot proteins including but not limited to TGFβ, PDGF, NGF, Veg1, bone morphogenic proteins, activin, inhibin and their analogs. The present invention also teaches that the N-terminal portions of the glycoprotein hormones can be modified without disrupting the activity of the protein.

The dimerization domain strategy can be used to prepare any heterodimeric analog of the glycoprotein hormones, glycoprotein hormone including hCG/hFSH and hCG/hTSH chimeras (31,33,48,52) and/or deglycosylated hormones including those missing oligosaccharides at one or more positions on the α- or β-subunits (10), truncated glycoprotein hormone subunits, glycoprotein hormone subunits containing the hCG carboxyl terminus (52), glycoprotein hormones from different vertebrates, glycoprotein hormones in which the subunits are derived from different species, and heterodimers of other members of the cysteine knot family. Most members of the cysteine knot family with the exception of the glycoprotein hormones contain their own dimerization domains that are included in the "pro" portion of the prohormone. Dimerization domains from these other cysteine knot proteins can be substituted for the Fos and Jun sequences described above to promote dimerization of the glycoprotein hormones. In addition, the use of Fos, Jun, or other dimerization domains will facilitate production of heterodimeric cysteine knot proteins. For example, inhibin is composed of an α- and a β-subunit. When these are expressed in the same cell there is the potential for the formation of activin, a ββ homodimer, particularly when the Fos dimerization sequence is added to the N-terminus of the β-subunit and the Jun dimerization sequence is added to the N-terminus of the α-subunit. Use of the Fos-Jun or other heterodimerization strategy outlined here is expected to reduce the formation of these homodimers and facilitate the production of heterodimers that inhibit rather than potentiate FSH secretion. It should also be noted that the furin cleavage site can

be replaced with other cleavage sites. In addition, residues such as glycine and/or serine can be inserted between the Fos or Jun and the furin cleavage sites to facilitate cleavage of the dimerization domain from the remainder of the heterodimer. The heterodimers produced by addition of the dimerization domains will retain the properties of the native or mutant hormones produced without the presence of the dimerization domains. The heterodimers produced by addition of the dimerization domains will also serve as useful antigens. Thus, when the dimerization domain is retained on the protein, its antigenicity will be enhanced. When the dimerization domain is used to produce a protein containing a subunit or part of a subunit from a different species, its antigenicity will also be enhanced.

Example 1

Preparation and characterization of Fos-Jun hCG, an hCG analog containing the dimerization domain of Fos attached to the N-terminus of the α-subunit and the dimerization domain of Jun attached to the N-terminus of the β-subunit.

The hCG β-subunit cDNA was modified to add an NheI restriction endonuclease site at the end of the leader sequence (Figure 1). This enabled insertion of the dimerization domains of Fos or Jun at the C-terminal end of the hCG β-subunit signal sequence. The modification was made by manipulating the sequence of the naturally occurring hCG β-subunit cDNA starting with vectors that have been described (31,32,42). The coding sequence of the resulting vector is shown in Figure 1. When this coding sequence is expressed in mammalian cells, the signal sequence (i.e., amino acids MEMFQGLLLLLLLSMGGTWA, single amino acid code) will be removed and like the hCG β-subunit, the resulting protein should have a serine residue at its N-terminus. The codons between the NheI and BamHI restriction sites shown in Figure 1 were replaced with bases that included the coding sequences for the portions of the Fos and Jun dimerization domains capable of forming heterodimers. This

created the constructs shown in Figures 2 and 3. The Fos construct was prepared by annealing oligonucleotides 1002 and 1003 (Figure 4) and filling them in with Vent Polymerase (New England Biolabs, Beverly, MA). Oligonucleotides 1004 and 1005 (Figure 4) were used in a polymerase chain reaction (PCR) with the filled-in product of oligonucleotides 1002 and 1003 to produce a DNA fragment approximately 175 base pairs long. This was purified by agarose gel electrophoresis, digested with NheI and BamHI and the large fragment that resulted was subcloned into the NheI-BamHI sites of the construct shown in Figure 1 to produce the construct shown in Figure 2. The Jun construct (Figure 3) was prepared in a similar fashion except that oligonucleotides 1006 and 1007 (Figure 4) were used for the fill-in reaction and oligonucleotides 1008 and 1009 (Figure 4) were used for PCR.

Following DNA sequencing to make certain that the complex having the desired codons had been obtained, the construct containing the Fos codons described in Figure 2 was digested with BsmI and BamHI. The short piece of DNA between these sites was replaced with the BsmI-BamHI digestion product of the human α-subunit cDNA to create the construct containing the codons illustrated in Figure 5. The final coding construct shown in Figure 5 was then subcloned into an expression vector (pCI') that was a modification of pCI, a vector obtained from Promega, Madison, WI and created pCI'-Fos-α. The modification of pCI to create pCI' consisted of moving the BamHI site from a region outside the polyadenylation signal to a position near the 3' end of the polylinker. This was accomplished using the polymerase chain reaction and was done to facilitate cloning the coding constructs into the expression vector. It is not necessary to use this vector to observe expression in mammalian cells. Virtually any vector capable of expressing proteins in mammalian cells will suffice including that known as pSVL, commercially available from Pharmacia Co., Piscataway, NJ. pSVL can be used directly without modification since it contains a polylinker with appropriately positioned XhoI and BamHI sites to facilitate the cloning and expression of the protein.

Using a similar strategy, a DNA construct that encodes a sequence having the hCG β-subunit leader, a segment related to the Jun oncoprotein, and the hCG β-subunit was prepared. The construct shown in Figure 3 was digested with BspMI and BamHI and the small fragment that was produced was replaced with the fragment obtained from pKBM-hCGβ' (31) by digestion with BanI and BamHI to create the construct shown in Figure 6. XhoI-BamHI fragment obtained from the resulting construct was ligated into the pCI' expression vector using the XhoI-BamHI sites to create pCI'-Jun-hCGβ'. As noted earlier, it would not be necessary to use pCI' for this purpose. pSVL has the appropriate restriction sites and would permit expression of the protein in COS-7 cells.

PCI'-Fos-α and pCI-Jun-hCGβ' were co-transfected into CHO cells using a calcium phosphate method as described (31) to cause the secretion of heterodimers comprised of Fos-α and Jun-hCGβ'. The heterodimers were recognized by monoclonal antibodies to the hCG α- and β-subunits A113 and B112, respectively (45,46). These specific antibodies are not required to measure the presence of heterodimers in the cell culture media and nearly any monoclonal or polyclonal antibodies that have epitopes on the surface of hCG α- and β-subunits that does not involve the N-terminus of the α- or β-subunits will be adequate. The Fos-α/Jun-hCGβ' heterodimer stimulated signal transduction (cyclic AMP accumulation) in CHO cells that express the rat LH receptor with approximately the same potency as hCG (Figure 7). This showed that the addition of the dimerization domains to the N-termini of both subunits did not adversely affect the biological activity of hCG.

This construct inhibited binding of 125I-hCG to cells that express LH receptors with approximately equal potency as hCG that had been purified from urine (Figure 8) indicating that the presence of the Fos and Jun sequences on the N-termini of the subunits did not interfere with receptor interaction. Methods for monitoring binding of

radioiodinated hCG to LH receptors have been described previously (31,33,45-48) and are well-known in the art.

Example 2

Preparation and characterization of Fos-Jun hCGαδ2, an hCG analog containing the dimerization domain of Fos attached to the N-terminus of the α-subunit lacking the glycosylation signal at α-subunit residue 52 and the dimerization domain of Jun attached to the N-terminus of the β-subunit.

Removal of the oligosaccharide normally found on the human α-subunit at residue 52 reduces the ability of the α-subunit analog to combine with the hCG β-subunit (10). This example shows that addition of the Fos dimerization domain to an α-subunit analog lacking the glycosylation signal responsible for adding an oligosaccharide at residue 52 (Fos-αδ52) facilitated formation of heterodimers with Jun-hCGβ'.

A construct encoding the human α-subunit lacking the glycosylation signal at residue 52 was prepared by PCR mutagenesis. Oligonucleotides 739 and 839 (Figure 4) were used as primers and pKBM-α (31) as template to create an α-subunit construct (pMB507) encoding a glutamine at residue 52 and to introduce BglII and SpeI restriction endonuclease sites. A second PCR was performed using oligonucleotides 850 and 851 (Figure 4) as primers and pKBM-α (31) as template to create an α-subunit construct encoding an aspartic acid at residue 52 and a glutamine at residue 78. The BglII-SpeI fragment of this construct was subcloned into the BglII-SpeI sites of pMB507 to create an α-subunit construct encoding an aspartic acid corresponding to human α-subunit residue 52. The coding sequence of this mutation (Figure 9) was confirmed by dideoxy DNA sequencing. Although this sequence also adds silent

restriction sites for BglII and SpeI to facilitate preparation of additional mutants, these are not needed to express an analog lacking the oligosaccharide at amino acid 52. Construction of the vector encoding Fos-αδ52 was similar to that encoding Fos-α except that the construct encoding the αδ52-subunit (Figure 9) was used in place of that encoding the α-subunit to create the sequence shown in Figure 10.

Co-expression of Fos-αδ52 and Jun-β subunits in COS-7 cells led to secretion of heterodimer that could be readily detected in a sandwich radioimmunoassay (41) using monoclonal antibodies A113 and B112 to the α- and β-subunits of hCG. Under these same conditions, much lower amounts of heterodimer were obtained by co-expression of αδ52 and hCG-β subunits lacking the Fos and Jun dimerization domain in COS-7 cells. This showed that the presence of the Fos and Jun sequences at the N-termini of the αδ52- and β-subunits facilitated dimer formation. These results are shown in Table 1.

Table 1

Formation of hCG heterodimers in which the α-subunit is missing an glycosylation signal needed for efficient subunit combination.

Subunit Combination	Amount of Heterodimer*
No heterodimer (blank)	2554 ± 215
hCG α + hCG β	15281 ± 493
Fos-αδ52 + Jun-hCGβ'	25506 ± 750

* Values represent the results of sandwich immunoassays in which the analyte was captured using an anti-α-subunit antibody (A113) and detected using a radioiodinated anti-β-subunit antibody (B112). The amount of culture media used in each assay was identical (50 µl). the higher the number of counts bound in the assay, the greater the amount of heterodimer. The presence of the Fos-Jun dimerization domain increased the ability of the deglycosylated α-subunit to combine with hCG β-subunit to at least as high a level as that seen for hCG.

Example 3

Preparation and characterization of Fos-Jun hCG analogs containing the dimerization domain of Fos attached to the N-terminus of the α-subunit and the dimerization domain of Jun attached to the N-terminus of the β-subunit lacking the seatbelt or with a seatbelt that cannot be latched.

The seatbelt is known to be essential for heterodimer formation. Mutations that disrupt the seatbelt or disrupt the seatbelt latch prevent subunit combination (49). Use of a dimerization domain can enable the formation of glycoprotein hormone analogs that lack the seatbelt and that are able to stimulate signal transduction.

The coding sequence of hCG β-subunit was modified to eliminate the seatbelt latch by replacing Cys26 with alanine. Some other analogs were prepared in which the coding sequence was truncated after the codon for amino acid 92. In addition, to increase the probability that the two subunits would be held together in the heterodimer lacking a properly closed seatbelt in the same orientation as they would be in hCG, an intersubunit disulfide was engineered between the two subunits. This disulfide was placed between the two cysteine knots corresponding to residue 31 from the α-subunit and residue 38 from the β-subunit. Residue 31 is already a cysteine in the α-subunit and normally forms a disulfide with the cysteine at α-subunit residue 7. Changing Cys7 to alanine, provided a free disulfide from the α-subunit for use in the intersubunit disulfide. It's counterpart in the β-subunit was created by changing Tyr37 to cysteine.

The coding sequences of Fos-αC7A, Jun-hCGβ'Y37C, Jun-hCGβ'Y37C/C26A, and Jun-hCGβ'Y37C/C26AδSeatbelt are illustrated in Figures 11,12,13, and 14, respectively. These were prepared using a combination of PCR and cassette mutagenesis starting with constructs that have already been described. Fos-αC7A (Figure 11) was prepared by taking advantage of the fact that the construct encoding

Fos-α contains HindIII and BspEI endonuclease restriction sites on either side of the codon corresponding to human α-subunit Cys7. Fos-αC7A was prepared by replacing the small HindIII-BspEI fragment of the coding vector for Fos-α with a cassette prepared from oligonucleotides 1053 and 1054 (Figure 4). The coding sequence of Jun-hCGβ'Y37C (Figure 12) was prepared by taking advantage of the fact that the pKBM-hCGβ' (31) contains NgoMI and PstI endonuclease restriction sites surrounding the codon to be changed (i.e., hCG β-subunit Tyr37). Jun-hCGβ'Y37C was prepared in two steps. First, the small NgoMI-PstI site in pKBM-hCGβ' was replaced with a cassette prepared by annealing oligonucleotides 845 and 877 (Figure 4). Second, the fragment of pKBM-hCGβ' between the BanI and BamHI sites was cloned into the BspMI-BamHI sites of the vector illustrated in Figure 3 to create the coding sequence of Jun-hCGβ'Y37C. The coding sequence of Jun-hCGβ'Y37C/C26A (Figure 13) was prepared using oligonucleotides 1026 and 1027 (Figure 4). These were annealed and filled-in to create a cassette having AvrII and NgoMI restriction sites at its ends. This was digested with AvrII and NgoMI and the resulting fragment was cloned into the AvrII-NgoMI fragment of Jun-hCGβ'Y37C. The coding sequence of Jun-hCGβ'Y37C/C26Aδ92 (Figure 14) was prepared by replacing the XhoI-PvuII fragment of a truncated hCG β-subunit construct with that from Jun-hCGβ'Y37C/C26A. The truncated hCG β-subunit construct had been prepared in two steps. First, the PvuII-BamHI fragment was replaced with a cassette prepared by annealing and filling-in oligonucleotides 435 and 436 (Figure 4). This cassette was digested with PvuII and BamHI sites and cloned into the PvuII-BamHI fragment of pKBM-hCG β' (31). This created a BssHII site at the codons for hCG β-subunit amino acids 90-92. The truncated hCG β-subunit was prepared by replacing the BssHII-BamHI fragment of this construct with a cassette prepared by annealing oligonucleotides 837 and 838 (Figure 4).

Fos-αC7A was co-expressed in CHO and/or COS-7 cells with Jun-hCGβ'Y37C, Jun-hCGβ'Y37C/C26A, or Jun-hCGβ'Y37C/C26Aδ92 to yield

heterodimers Fos-Jun-hCG-SS, Fos-Jun-hCG-SS-δlatch, or Fos-Jun-hCG-SS-δseatbelt. The heterodimers were readily detected in assays employing A113 and ^{125}I-B112 for capture and detection, respectively. The activities of Fos-Jun-hCG-SS and Fos-Jun-hCG-SS-δseatbelt were determined in signal transduction assays (Figure 7). The presence of the second intersubunit disulfide in Fos-Jun-hCG-SS reduced the activity of Fos-Jun-hCG slightly in signal transduction assays (Figure 7) and in receptor binding assays (Figure 8) as can be seen by comparing the activities of and Fos-Jun-hCG in both assays. The analog lacking the seatbelt was much less active than that of hCG, Fos-Jun-hCG, and Fos-Jun-hCG-SS indicating that the seatbelt had a substantial influence on the activities of hCG in these assays. However, the presence of the Fos-Jun dimerization enabled production of sufficient heterodimer to be able to detect the activities of the material lacking the seatbelt. Without a dimerization domain, it would have been nearly impossible to prepare sufficient heterodimer lacking the seatbelt to test its activity.

These analogs were also tested in FSH and TSH signal transduction assays. hCG has very low ability to stimulate signal transduction in cells containing FSH or TSH receptors (50). The presence of Fos and Jun did not increase the ability of hCG to elicit signal transduction in cells expressing either FSH (Figures 15) or TSH receptors (Figure 16). Thus, the presence of the dimerization domain did not alter receptor specificity. In addition, the presence of the disulfide did not influence receptor specificity. The analog lacking the seatbelt had low activity, however, its activity in the FSH and TSH assays was only slightly lower than its activity in the LH receptor signal transduction assays. This shows that the presence of the dimerization domain would not be expected to alter receptor interaction or specificity. However, removing the seatbelt increased the maximal amount of signal transduction that could be obtained in TSH assays (Figure 16).

Example 4

Fos-Jun hCG analogs containing domains that can be cleaved during synthesis in eucaryotic cells.

The dimerization domains of the analogs whose sequences are described in Figures 5, 6, and 9 remain associated with the heterodimers. In some cases such as when the heterodimer is to be used as an antigen or a pharmaceutical compound, it may be desirable to remove the dimerization domain. This can be accomplished by a variety of proteolytic methods including digestion with aminopeptidases and/or endopeptidases. In the latter case it usually necessary to incorporate a specific endopeptidase restriction site between the dimerization sequence and the protein to be produced. Many of these are well-known in the art and include the sequences recognized by enterokinase (i.e., DDDDK, single letter amino acid code) and FactorXa (i.e., IEGR, single letter amino acid code). It is also possible to include a furin cleavage sequence in this location as illustrated in Figure 17. The protein heterodimer to be produced is expected to form a heterodimer in the lumen of the endoplasmic reticulum and then be cleaved by a furin protease located in a downstream part of the secretion pathway. This strategy has the advantage in that it does not require protease digestion of the secreted product.

Example 5

Addition of dimerization domains to other glycoprotein hormones.

Fos-Jun constructs similar to those described in Example 1 can be prepared from other glycoprotein hormone α- and β-subunits including those of hLH, hFSH, hTSH and other vertebrate glycoprotein hormones. Figure 17 lists the amino acid sequences of some of these with the presence of the furin cleavage site. Addition of

the N-terminal dimerization domains would be expected to increase the efficiency of heterodimer formation, particularly with α- and β-subunit analogs that do not readily dimerize. Production and analysis of these analogs would be similar to that of Fos-Jun hCG. It would involve their expression in eucaryotic cells, measurement in sandwich immunoassays using antibodies to the α-subunit for capture and radiolabeled antibodies to the β-subunit for detection, and assay using CHO cells expressing LH, FSH, or TSH receptors. By analogy to Example 4, it should be possible to include endopeptidase sites to cleave the dimerization domain. It should be noted that the location of the furin site shown is not essential to produce these proteins as heterodimers.

Example 6

Addition of different dimerization domains to enhance the formation of glycoprotein hormone heterodimers.

It is not necessary to use the Fos-Jun dimerization strategy to enhance the formation of heterodimers. Addition of nearly any other dimerization domains should suffice. This includes any coiled-coil pair that forms heterodimers. These would be introduced onto the α- and β-subunits of the glycoprotein hormones in a fashion similar to that used to prepare the Fos-Jun analogs. Alternatively, it would be possible to modify the sequences of the Fos and Jun sequences to create additional coiled-coils. Methods for producing and aligning coiled-coils are well known in the art (51). One could also use the heavy and light chains of the immunoglobulins to promote dimerization. An example of the use of the light chain coupled to the α-subunit and the heavy chain coupled to the β-subunit is illustrated in Figures 18 and 19. This could also be reversed such that the light chain is coupled to the β-subunit and the heavy chain is coupled to the α-subunit. Since the heavy and light chains of the antibodies can be selected to bind to various targets, this approach has the additional advantage of

enabling one to direct the heterodimers to particular tissues that contain binding sites for the antibodies. By incorporating an enzyme cleavage site, it would be possible to obtain selected release of the hormones from the antibodies at the site in which they had been localized by the presence of the immunoglobulins. The sequences shown in Figures 18 and 19 illustrate furin cleavage sites between the immunoglobulin domains and the α- and β-subunits. These could be replaced by any target-specific cleavage site to promote release of the heterodimeric glycoprotein hormone or hormone analog at a desired location. Further, it would be possible to include a furin cleavage site in only the light chain - α-subunit construct or the heavy chain - β-subunit construct and to include a target-specific cleavage site in the other subunit construct at the location shown in Figures 18 or 19 by the furin sites. It should be noted that the constructs illustrated in Figure 18 will be crosslinked by a disulfide near the junction of the immunoglobulin constant domains with the α- and β-subunits. This disulfide may constrict the conformation of the subunits and reduce their activities. To eliminate this, the cysteine near this junction should be replaced with an alanine. Finally, it should be noted that the immunoglobulins illustrated are from the mouse. This would not be expected to be a requirement for the immunoglobulin domain to facilitate subunit combination or targeting. Thus, one could use immunoglobulin domains from other proteins and from other species. The use of immunoglobulin domains from humans would be an advantage for preparing glycoprotein hormone homodimers for use in humans. These domains would also be expected to enhance the half-lives of hormone analogs such as hLH that are known to have short half-live

Example 7

Addition of a dimerization domain at the C-terminus of the β-subunit that can form a heterodimer with a dimerization domain attached to the N-terminus of the α-subunit.

Another method of forming heterodimers of the glycoprotein hormones involves addition of the coding region for a dimerization domain such as the sequence needed for dimerization of Fos or Jun or any other protein heterodimer to the C-terminus of the β-subunit unit. When this protein is expressed with a protein that has a complementary dimerization domain attached to the N-terminal end of the α-subunit, it will also form a heterodimer. An example of a β-subunit analog that can enhance dimerization of hCG analogs that would otherwise dimerize poorly is illustrated in Figure 20. This illustrates the hCG β-subunit sequence containing a Jun dimerization domain at its C-terminus. When expressed with an α-subunit construct that encodes a Fos dimerization domain at its N-terminus this will lead to the formation of a heterodimer capable of interacting with LH receptors. α-subunit constructs capable of dimerizing with the β-subunit sequences illustrated in Figure 20 are illustrated in Figures 5 and 10. These α-constructs have the potential to form an intersubunit disulfide bond with the last two β-subunit Jun analogs illustrated in Figure 20. When this intersubunit bond is not desired, it can be eliminated by removing the codons for the amino acid sequence Cys-Gly-Gly (CGG). It should be noted that it is not necessary to use Fos and Jun sequences to augment dimerization. It should also be noted that because the C-terminus of the glycoprotein hormone β-subunits is not needed for their hormone activities and that the N-terminus of the glycoprotein hormone α-subunits is not needed for their hormone activities, this process could be applied to all the glycoprotein hormones. It should also be noted that the presence of the hCG β-subunit C-terminus can be used to extend the half-life of all the circulating hormones. Thus, it would be expected that addition of the C-terminus of the hCG β-subunit to the C-

terminus of the other glycoprotein hormone β-subunits would be useful in preparing this type of heterodimers and eliciting heterodimers that would have prolonged half-lives. It would also be expected that heterodimers in which the dimerization domain was crosslinked by a disulfide bond could be prepared by expressing the α-subunit constructs illustrated in Figures 5 or 10 with either of the last two β-subunit constructs illustrated in Figure 20. This would be expected to stabilize the heterodimer.

Figure 1 illustrates the coding sequence of the hCG β-subunit cDNA between the XhoI and BamHI sites except that the codons for amino acids 2-8 have been deleted. This sequence contains an NheI restriction site between the codons for alanine and serine (i.e., codons -1 and +1, respectively) and is similar to that described by Slaughter et al. (31). The leader sequence from this vector encodes the same amino acids found in hCG β-subunit and was used to prepare subsequent expression constructs because it enables the proteins to enter the secretion pathway. It should be noted that it is not necessary to use the hCG β-subunit leader for this purpose. Most other known leader sequences can be used to drive secretion.

Figure 2 illustrates the coding sequence of a vector that encodes the hCG β-subunit signal sequence upstream (5') of bases that encode serine, cysteine, two glycines, and portions of the Fos dimerization domain (one letter code). While this sequence serves well to elicit formation of homodimers, other sequences are expected to work equally well. Indeed, it is expected that most other amino acid sequences known to participate in heterodimer formation will function as a dimerization domain similar to the sequence illustrated in Figure 2. Dimerization domains can be designed to be similar to proteins or portions of proteins that are known to form coiled coils. They can be derived from other proteins known to form heterodimers such as portions of the heavy and light chains of immunoglobulins. It is not essential to use the sequence "cysteine-glycine-glycine" shown at the N-terminus of the protein in Figure 2. The sequence "glycine-glycine-cysteine" can also be incorporated at the C-terminal

end of the Fos dimerization domain such that the cysteine residue would occupy the position normally occupied by glutamine at residue 5 of the human α-subunit. The cysteine has been incorporated to promote the formation of a disulfide to stabilize the heterodimer.

Figure 3 illustrates the coding sequence of a vector that encodes the hCG β-subunit signal sequence upstream (5') of bases that encode serine, cysteine, two glycines, and portions of the Jun dimerization domain (one letter code). While this sequence serves well to elicit formation of homodimers, other sequences are expected to work equally well. Indeed, it is expected that most other amino acid sequences known to participate in heterodimer formation will function as a dimerization domain similar to the sequence illustrated in Figure 3. Dimerization domains can be designed to be similar to proteins or portions of proteins that are known to form coiled coils. They can be derived from other proteins known to form heterodimers such as portions of the heavy and light chains of immunoglobulins. It is not essential to use the sequence "cysteine-glycine-glycine" shown at the N-terminus of the protein in Figure 3. The sequence "glycine-glycine-cysteine" can also be incorporated at the C-terminal end of the Jun dimerization domain such that the cysteine residue would occupy the position normally occupied by glutamine at residue 6 of the human β-subunit. The cysteine has been incorporated to promote the formation of a disulfide to stabilize the heterodimer.

Figure 4 illustrates the sequences of oligonucleotides used in this work including primers used to create a cassette that permitted insertion of the Fos and Jun dimerization domain amino acid coding sequences into the construct illustrated in Figure 1. Each oligonucleotide was synthesized by standard methods. Oligonucleotides 1002 and 1003 (shown in the positions that they hybridize) were mixed and heated to 95°C for 2 minutes. Vent DNA polymerase (New England Biolabs, Beverly, MA) was added and the reaction allowed to cool slowly to 68°C and

maintained at that temperature for 5 min to permit the fill-in reaction to occur. An aliquot of the reaction was added to a second tube containing primers 1004 and 1005 and the tube was heated to 95°C for 2 minutes. Vent DNA polymerase was added and the reaction allowed to cycle repeatedly between 55°C (30 seconds), 72°C (30 seconds), and 95°C (30 seconds). After 20 cycles, the DNA was removed and purified by electrophoresis through 2% agarose gels. The band at approximately 174 base pairs was electroeluted from the gel, ethanol precipitated, and digested with NheI and BamHI endonucleases. The resulting fragment was ligated into a vector containing the construct illustrated in Figure 1 at the NheI and BamHI sites. Similarly, oligonucleotides 1006 and 1007 (shown in the positions that they hybridize) were mixed and heated to 95°C for 2 minutes. Vent DNA polymerase (New England Biolabs) was added and the reaction allowed to cool slowly to 68°C and maintained at that temperature for 5 min to permit the fill-in reaction to occur. An aliquot of the reaction was added to a second tube containing primers 1008 and 1009 and the tube was heated to 95°C for 2 minutes. Vent DNA polymerase was added and the reaction allowed to cycle repeatedly between 55°C (30 seconds), 72°C (30 seconds), and 95°C (30 seconds). After 20 cycles, the DNA was removed and purified by electrophoresis through 2% agarose gels. The band at approximately 174 base pairs was electroeluted from the gel, ethanol precipitated, and digested with NheI and BamHI endonucleases. The resulting fragment was ligated into a vector containing the construct illustrated in Figure 1 at the NheI and BamHI sites. Use of the other oligonucleotides is described in the Examples.

Figure 5 shows the coding sequence of the entire Fos-hCG-α-subunit construct. It should be noted that it differs from the native hCG α-subunit by the presence of the hCG β-subunit leader sequence, the presence of the Fos sequence, and the lack of 4 α-subunit amino acids, namely Ala1-Pro2-Asp3-Val4. These residues were omitted to facilitate formation of the heterodimer. However, it is possible to retain these residues by inserting a linker between the Fos and α-subunit coding regions. When this linker

contains a protease cleavage recognition site (e.g., amino acids arginine-serine-lysine-arginine) and when a similar protease cleavage recognition site is introduced between the Jun sequence and the β-subunit construct described later, the heterodimer that is created will lack its Fos sequence at the N-terminus.

Figure 6 shows the coding sequence of the entire Jun-hCGβ'-subunit construct. It should be noted that it differs from the native hCG β-subunit by the presence of the Jun sequence and the lack of 6 β-subunit amino acids, namely Ser1-Lys2-Glu3-Pro4-Leu5-Arg6. These residues were omitted to facilitate formation of the heterodimer. However, it is possible to retain these residues by inserting a linker between the Jun and β-subunit coding regions. When this linker contains a protease cleavage recognition site and when a similar protease cleavage recognition site is introduced between the Fos sequence and the α-subunit construct described earlier, the heterodimer that is created will lack its Jun sequence at the N-terminus.

Figure 7 shows that the heterodimer containing the Fos-Jun amino acid sequences at its N-termini can stimulate signal transduction in a similar fashion as hCG. Signal transduction (production of cyclic AMP) was monitored using CHO cells that express rat LH receptors as described (33).

Figure 8 shows that the heterodimer containing the Fos-Jun amino acid sequences at its N-termini can inhibit the binding of radioiodinated hCG to CHO cells expressing rat LH receptors in a similar fashion as hCG. Receptor binding was monitored by inhibiting the binding of radioiodinated hCG to cells expressing LH receptors as described (33).

Figure 9 illustrates the sequence of an α-subunit construct that is lacking the glycosylation signal normally found at Asn52 of the mature human α-subunit. This construct can be prepared by anyone skilled in the art of DNA mutagenesis by using

polymerase chain reaction or other mutagenesis to introduce BglII and SpeI endonuclease restriction sites into the cDNA for the human α-subunit. This will permit making of the construct illustrated here by cassette mutagenesis between the BglII and SpeI sites. Elimination of the glycosylation signal is illustrated here by replacing the codon for Asn52 with that of Asp. Constructs with this mutation are known to have reduced efficacy for LH receptors (10). However, it is not essential that this specific mutation be prepared to eliminate the oligosaccharide at this location..

Figure 10 illustrates the sequence of the Fos-α-subunit construct that is lacking the glycosylation signal corresponding to human α-subunit residue 52 caused by substitution of an aspartic acid residue for the asparagine normally found at this residue of the human α-subunit. Unlike human α-subunit that has been deglycosylated by this mutation that combines with hCG β-subunit poorly, the construct shown here combines well with the Jun-hCGβ'-subunit to form a heterodimer that binds to LH receptors.

Figure 11 illustrates the sequence of the Fos-α-subunit construct in which the cysteine normally found at position 7 of the human α-subunit has been replaced by an alanine. When expressed with the Jun-hCGβ'-subunit construct shown in Figure 12, this will cause the formation of an intersubunit disulfide between residues of the cysteine knots.

Figure 12 illustrates the sequence of the Jun-hCGβ'-subunit in which the tyrosine normally found in the hCG β-subunit at residue 37 has been replaced by a cysteine. When expressed with the construct illustrated in Figure 11, the heterodimer that is formed will have an intersubunit between residues of its cysteine knots.

Figure 13 illustrates the Jun-hCGβ'-Y37C-C26A construct. This construct is similar to that in Figure 12 except that the codon for cysteine normally found at residue

26 in the hCG β-subunit has been changed to alanine. This will prevent closure of the seatbelt. Expression of this construct along with the construct illustrated in Figure 11 was shown to cause the formation of a heterodimer even though the heterodimer is unable to latch the seatbelt loop.

Figure 14 illustrates the Jun-hCGβ'-Y37C-C26A-δ92 construct. This construct is similar to that in Figure 13 except that the codons for all hCG β-subunit seatbelt residues except 91 and 92 are missing. This construct is also missing the residues in the C-terminus normally found in the hCG β-subunit. Expression of this construct along with the construct illustrated in Figure 11 was shown to cause the formation of a heterodimer even though the heterodimer lacked the seatbelt loop.

Figure 15 illustrates the abilities of hFSH, hCG, Fos-Jun-hCG-SS/δseatbelt, and Fos-Jun-hCG-SS to stimulate signal transduction in CHO cells expressing human FSH receptors. This shows that hCG is much less potent than hFSH as expected. However, the absence of the seatbelt causes only a small additional influence on the activity of hCG.

Figure 16 illustrates the abilities of bovine TSH, hCG, Fos-Jun-hCG-SS/δseatbelt, and Fos-Jun-hCG-SS to stimulate signal transduction in CHO cells expressing human FSH receptors. This shows that hCG is much less potent than TSH as expected. However, the absence of the seatbelt causes only a small additional influence on the activity of hCG.

Figure 17 illustrates the sequences of constructs of Fos-hCG α-subunit containing a furin cleavage site and the sequences of Jun-hCG β-subunit, Jun-hLH β-subunit, Jun-hFSH β-subunit, Jun-hTSH β-subunit, Jun-hCG/hFSH β-subunit chimera, and Jun-hCG/hTSH β-subunit chimera containing a furin cleavage site. Expression of

the α-subunit constructs with the β-subunit constructs is expected to lead to the formation of α/β heterodimers similar to the formation of Fos-Jun-hCG and analogs of Fos-Jun-hCG. Following subunit combination in the lumen of the endoplasmic reticulum, the Fos and Jun dimerization domains are expected to be removed during passage of the heterodimer through the Golgi apparatus and trans-Golgi network as it is being readied for secretion. These constructs can be prepared following the steps used to prepare Fos-α and Jun-β except that the codons for the amino acids in a furin cleavage site (e.g., KSKR) are inserted into the oligonucleotides used to encode the Fos and Jun sequences (i.e., the sequences illustrated in Figure 3 and Figure 7). The internal hyphens are included to facilitate identification of the portions of the molecule that correspond to the hCG β-subunit leader, Fos or Jun dimerization domains, furin cleavage signal, and mature protein. The hyphens at the ends of the lines indicate that the sequence continues uninterrupted on the next line.

Figure 18 illustrates the sequences of constructs containing immunoglobulin dimerization domains at their N-termini. When the α-subunit construct and a β-subunit construct are expressed in the same cell, an α/β heterodimer will be formed. When the furin cleavage site is present as illustrated in these sequences, the immunoglobulin domains will be removed. Inclusion of the furin cleavage site in only the α- or β-construct is expected to create a dimer that is held to the immunoglobulin domains through bonds to one subunit.

Figure 19 illustrates the sequences of constructs containing immunoglobulin dimerization domains at their N-termini. Unlike those in Figure 18, these immunoglobulin dimerization domains will not form an intersubunit disulfide.

Figure 20 illustrates the amino acid sequences of β-subunit constructs containing the dimerization domain from Jun at the carboxyterminus of their

dimerization domains. As noted, some of these have the ability to form a disulfide crosslink between the two subunits.

Throughout this application, various publications have been referenced. The disclosures in these publications are incorporated herein by reference in order to more fully describe the state of the art.

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The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.

I claim:

1. A method for forming a subunit combination of a cysteine knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of:

(a) attaching a dimerization domain to the amino termini of both an α-subunit and a β-subunit of a cysteine knot protein; and

(b) dimerizing the α-subunit and β-subunit to form a heterodimeric protein analog.

2. The method according to claim 1, wherein a Fos dimerization sequence domain is attached to the amino-terminus of the β-subunit and a Jun dimerization sequence domain is attached to the amino-terminus of the α-subunit.

3. The method according to claim 1, wherein a Fos dimerization sequence domain is attached to the amino-terminus of the α-subunit and a Jun dimerization sequence domain is attached to the amino-terminus of the β-subunit.

4. The method according to claim 2, wherein a glycine or serine residue is inserted between the Fos or Jun dimerization sequence domain and a furin cleavage site to facilitate cleavage of the dimerization sequence domain from the heterodimer.

5. The method according to claim 3, wherein a glycine or serine residue is inserted between the Fos or Jun dimerization sequence domain and a furin cleavage site to facilitate cleavage of the dimerization sequence domain from the heterodimer.

6. The method according to claim 1, wherein the dimerization domain is a heavy or light chain of an immunoglobulin.

7. The method according to claim 1, wherein the heterodimeric protein analog is a glycoprotein hormone heterodimer selected from the group consisting of hCG, hLH, hFSH, hTSH, TGFβ, PDGF, NGF, Veg1, bone morphogenic proteins, activin, inhibin, and analogs thereof.

8. The method according to claim 1, wherein the heterodimeric protein analog is selected from the group consisting of hCG/hFSH chimeras, hCG/hTSH chimeras, deglycosylated hormones, truncated glycoprotein hormones, mutant glycoprotein hormones, and glycoprotein hormones containing an hCG carboxyl terminus.

9. The method according to claim 1, further comprising incorporating protease cleavage sites between additional N-terminal sequences and the α-subunit and the β-subunit of the cysteine knot protein to remove the dimerization domains from the heterodimeric protein analog.

10. The method according to claim 1, wherein the cysteine knot protein is a glycoprotein hormone heterodimer having the oligosaccharide genetically removed from the α-subunit at Asn52.

11. The method according to claim 1, wherein the cysteine knot protein is a glycoprotein hormone heterodimer lacking a seatbelt.

12. A method for forming a subunit combination of a cysteine knot protein having an α-subunit and a β-subunit to prepare a heterodimeric protein analog which comprises the steps of:

(a) attaching a dimerization domain to the amino terminus of an α-subunit and the carboxy terminus of a β-subunit of a cysteine knot protein; and

(b) dimerizing the α-subunit and β-subunit to form a heterodimeric protein analog.

13. The method according to claim 12, wherein a Fos dimerization sequence domain is attached to the carboxy-terminus of the β-subunit and a Jun dimerization sequence domain is attached to the amino-terminus of the α-subunit.

14. The method according to claim 12, wherein a Fos dimerization sequence domain is attached to the amino-terminus of the α-subunit and a Jun dimerization sequence domain is attached to the carboxy terminus of the β-subunit.

15. The method according to claim 13, wherein a protease cleavage site is inserted between the dimerization sequence domain and the α-subunit and a protease cleavage site is inserted between the dimerization sequence domain and the β-subunit.

16. The method according to claim 15, wherein the protease cleavage site is furin.

17. The method according to claim 12, wherein the dimerization domain is a heavy or light chain of an immunoglobulin.

18. The method according to claim 12, wherein the heterodimeric protein analog is selected from the group consisting of hCG/hFSH chimeras, hCG/hTSH chimeras, deglycosylated hormones, truncated glycoprotein hormones, mutant glycoprotein hormones, and glycoprotein hormones containing an hCG carboxyl terminus.

19. The method according to claim 12, wherein the cysteine knot protein is a glycoprotein hormone heterodimer having the oligosaccharide genetically removed from the α-subunit at Asn52.

Figure 1

```
                                    -20                 -10
                                     M  E  M  F  Q  G  L  L  L  L  L  L  L  S  M  G  G  T  W
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGAGCATGGGCGGGACATGG-3
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACACGACGACTCGTACCCGCCCTGTACC-5
    XhoI  XbaI

   -1  1    10                  20                  30                  40
    A  S  C  R  P  I  N  A  T  L  A  V  E  K  E  G  C  P  V  C  I  T  V  N  T  T  I  C  A  G  Y  C  P  T
5'-GCTAGCTGCCGCCCCATCAATGCCACCCTGGCTGTGGAGAAGGAGGGCTGCCCCGTGTGCATCACCGTCAACACCACCATCTGTGCCGGCTACTGCCCCACC-3
3'-CGATCGACGGCGGGGTAGTTACGGTGGGACCGACACCTCTTCCTCCCGACGGGGCACACGTAGTGGCAGTTGTGGTAGACACGGCCGATGACGGGGTGG-5'
    NheI

                                    50                  60                  70
    M  T  R  V  L  Q  G  V  L  P  A  L  P  Q  V  V  C  N  Y  R  D  V  R  F  E  S  I  R  L  P  G  C  P  R
5'-ATGACCCGCGTGCTGCAGGGCGTCCTCCCGGCCCTGCCTCAGGTGGTGTGCAACTATCGCGATGTGCGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCGCGC-3'
3'-TACTGGGCGCACGACGTCCCGCAGGAGGGCCGGGACGGAGTCCACCACACGTTGATAGCGCTACACGCGAAGCTCAGGTAGGCCGAGGGACCGACGGGCGCG-5'

                                    80                  90                  100
     G  V  N  P  V  V  S  Y  A  V  A  L  S  C  Q  C  A  L  C  R  R  S  T  T  D  C  G  G  P  K  D  H  P  L
5'-GGCGTGAACCCCGTGGTCTCCTACGCCGTGGCTCTCAGCTGTCAATGTGCACTCTGCCGCCGCAGCACCACTGACTGCGGGGGTCCCAAGGACCACCCCTTG-3'
3'-CCGCACTTGGGGCACCAGAGGATGCGGCACCGAGAGTCGACAGTTACACGTGAGACGGCGGCGTCGTGGTGACTGACGCCCCCAGGGTTCCTGGTGGGGAAC-5'

    110                 120                 130                 140
     T  C  D  D  P  R  F  Q  D  S  S  S  S  K  A  P  P  S  L  P  S  P  S  R  L  P  G  P  S  D  T  P  I
5'-ACCTGTGATGACCCCCGCTTCCAGGACTCCTCTTCCTCAAAGGCCCCTCCCCCCAGCCTCCCAAGCCCATCCCGACTCCCGGGGCCCTCGGACACCCCGATC-3'
3'-TGGACACTACTGGGGGCGAAGGTCCTGAGGAGAAGGAGTTTCCGGGGAGGGGGGTCGGAGGGTTCGGGTAGGGCCCCGGGAGCCTGTGGGGCTAG-5'

    145
     L  P  Q  *
5'-CTCCCACAATAAAGGCTTCTCAATCCGCAAGCTGGGGAGCTCGGATCCGCGCGCGGTCGACCCGCGGAGCTCGGATCC-3'
3'-GAGGGTGTTATTTCCGAAGAGTTAGGCGTTCGACCCCTCGAGCCTAGGCGCGCGCAGCTGGGCGCCTCGAGCCTAGG-5'
                                                                SstI  BamHI
```

Figure 2

```
-20                                   -10
                                                            M   E   M   F   Q   G   L   L   L   L   L   L   L   S   M   G   G   T   W
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGCGGGACATGG-3
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACGACAACGACGACGACTCGTACCCGCCCTGTACC-5
    XhoI   XbaI

  -1  1                                10                                    20                                   30
    A   S   C   G   G   L   T   D   T   L   Q   A   E   T   D   Q   L   E   D   K   K   S   A   L   Q   T   E   I   A   N   L   L   K   E
5'-GCTAGCTGTGGTGGGTTAACCGATACCCTGCAAGCTGAAACTGATCAACTGGAAGATAAGAAATCTGCTCTGCAAACTGAAATCGCTAATCTGCTGAAAGAG-3'
3'-CGATCGACACCACCCAATTGGCTATGGGACGTTCGACTTTGACTAGTTGACCTTCTATTCTTTAGACGAGACGTTTGACTTTAGCGATTAGACGACTTTCTC-5'
    NheI

                        40                                    50
    K   E   K   L   E   F   I   L   A   G   Q   D   C   P   E   C   T   L   Q
5'-AAGGAAAAGCTTGAGTTCATCCTGGCCGGCCAAGATTGTCCGGAATGCACGCTACAGGGATCC-5'
3'-TTCCTTTTCGAACTCAAGTAGGACCGGCCGGTTCTAACAGGCCTTACGTGCGATGTCCCTAGG-3'
          HindIII              NgoMI              BspEI  BsmI            BamHI
```

Figure 3

```
                                    -20                 -10
                                     M  E  M  F  Q  G  L  L  L  L  L  L  S  M  G  G  T  W
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGAGCATGGGCGGGACATGG-3
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACAACGACGACTCGTACCCGCCCTGTACC-5
    XhoI  XbaI

                                -1  1                   10                  20                  30
                                 A  S  C  G  G  G  R  I  A  R  L  E  E  K  V  K  T  L  K  A  Q  N  S  E  L  A  S  T  A  N  M  L  R  E  Q
5'-GCTAGCTGTGGCGGCCGCATTGCTAGATTGGAAGAGAAAGTTAAAACTCTGAAGGCCCAAAACAGCGAACTGGCTTCCACTGCTAATATGCTGCGTGAACAA-3'
3'-CGATCGACACCGCCGGCGTAACGATCTAACCTTCTCTTTCAATTTTGAGACTTCCGGGTTTTGTCGCTTGACCGAAGGTGACGATTATACGACGCACTTGTT-5'
   NheI

                                                    40                  50
                                     V  A  Q  L  K  Q  K  V  M  G  L  R  P  R  C  L  S  R  *
5'-GTCGCTCAACTGAAGCAAAAGGTTATGGGTTTGCGCCCTAGGTGCCTTAGCAGGTAAGGATCC-3'
3'-CAGCGAGTTGACTTCGTTTTCCAATACCCAAACGCGGGATCCACGGAATCGTCCATTCCTAGG-5'
                                                       AvrII       BspMI    BamHI
```

Figure 4

Figure 5

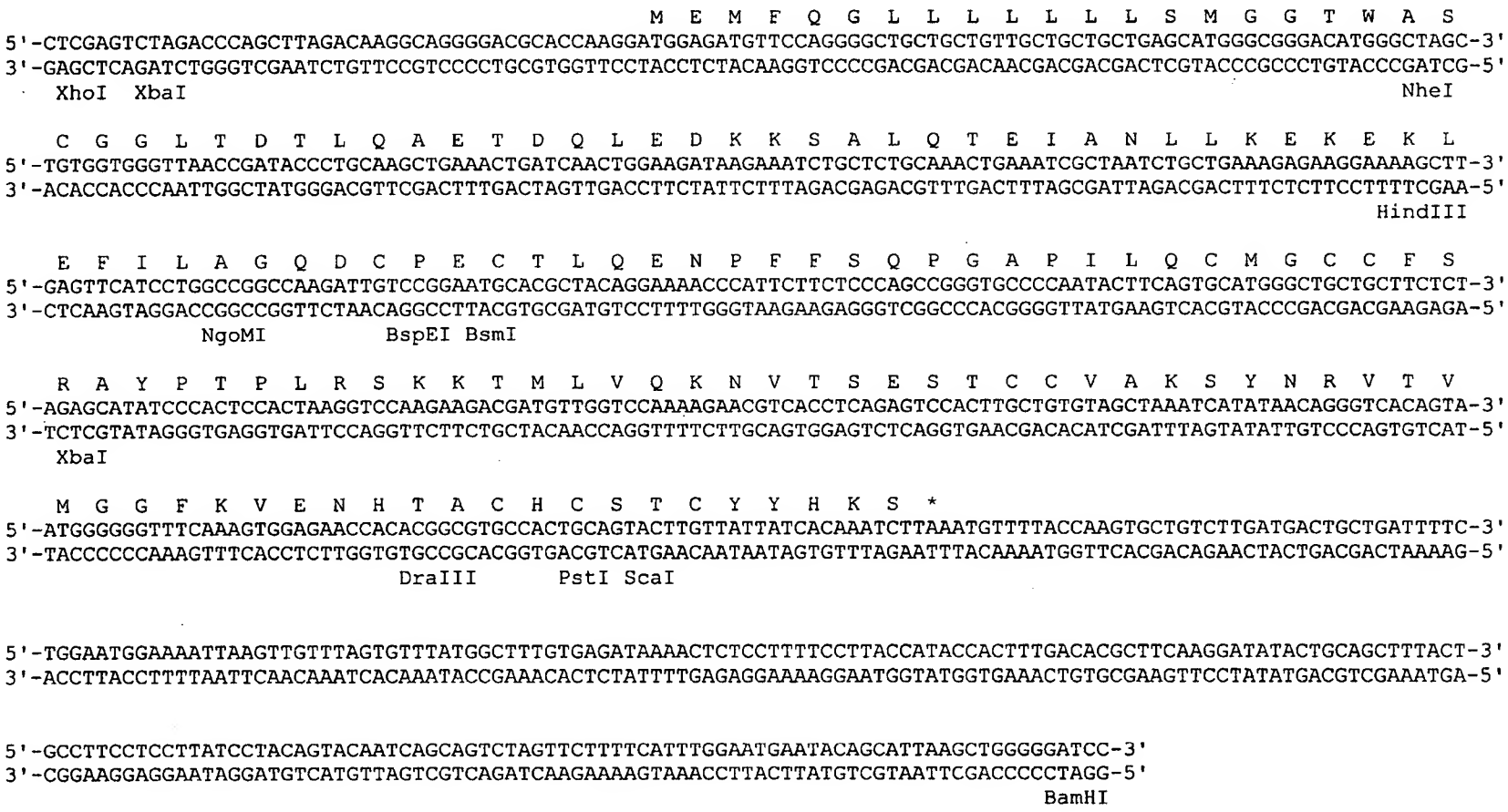

Figure 6

```
M  E  M  F  Q  G  L  L  L  L  L  L  S  M  G  G  T  W
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGCGGGACATGG-3'
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACGACAACGACGACGACTCGTACCCGCCCTGTACC-5'
    XhoI  XbaI

   A  S  C  G  G  R  I  A  R  L  E  E  K  V  K  T  L  K  A  Q  N  S  E  L  A  S  T  A  N  M  L  R  E  Q
5'-GCTAGCTGTGGCGGCCGCATTGCTAGATTGGAAGAGAAAGTTAAAACTCTGAAGGCCCAAAACAGCGAACTGGCTTCCACTGCTAATATGCTGCGTGAACAA-3'
3'-CGATCGACACCGCCGGCGTAACGATCTAACCTTCTCTTTCAATTTTGAGACTTCCGGGTTTTGTCGCTTGACCGAAGGTGACGATTATACGACGCACTTGTT-5'
    NheI

   V  A  Q  L  K  Q  K  V  M  G  L  R  P  R  C  R  P  I  N  A  T  L  A  V  E  K  E  G  C  P  V  C  I  T
5'-GTCGCTCAACTGAAGCAAAAGGTTATGGGTTTGCGCCCTAGGTGCCGCCCCATCAATGCCACCCTGGCTGTGGAGAAGGAGGGCTGCCCCGTGTGCATCACC-3'
3'-CAGCGAGTTGACTTCGTTTTCCAATACCCAAACGCGGGATCCACGGCGGGGTAGTTACGGTGGGACCGACACCTCTTCCTCCCGACGGGGCACACGTAGTGG-5'
                                    AvrII BanI

   V  N  T  T  I  C  A  G  Y  C  P  T  M  T  R  V  L  Q  G  V  L  P  A  L  P  Q  V  V  C  N  Y  R  D  V
5'-GTCAACACCACCATCTGTGCCGGCTACTGCCCCACCATGACCCGCGTGCTGCAGGGCGTCCTCCCGGCCCTGCCTCAGGTGGTGTGCAACTACCGCGATGTG-3'
3'-CAGTTGTGGTGGTAGACACGGCCGATGACGGGGTGGTACTGGGCGCACGACGTCCCGCAGGAGGGCCGGGACGGAGTCCACCACACGTTGATGGCGCTACAC-5'

   R  F  E  S  I  R  L  P  G  C  P  R  G  V  N  P  V  V  S  Y  A  V  A  L  S  C  Q  C  A  L  C  R  R  S
5'-CGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCGCGCGGCGTGAACCCCGTGGTCTCCTACGCCGTGGCTCTCAGCTGTCAATGTGCACTCTGCCGCCGCAGC-3'
3'-GCGAAGCTCAGGTAGGCCGAGGGACCGACGGGCGCGCCGCACTTGGGGCACCAGAGGATGCGGCACCGAGAGTCGACAGTTACACGTGAGACGGCGGCGTCG-5'

   T  T  D  C  G  G  P  K  D  H  P  L  T  C  D  D  P  R  F  Q  D  S  S  S  S  K  A  P  P  P  S  L  P  S
5'-ACCACTGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAGGACTCCTCTTCCTCAAAGGCCCCTCCCCCCAGCCTTCCAAGC-3'
3'-TGGTGACTGACGCCCCCAGGGTTCCTGGTGGGGAACTGGACACTACTGGGGGCGAAGGTCCTGAGGAGAAGGAGTTTCCGGGGAGGGGGGTCGGAAGGTTCG-5'

   P  S  R  L  P  G  P  S  D  T  P  I  L  P  Q  *
5'-CCATCCCGACTCCCGGGGCCCTCGGACACCCCGATCCTCCCACAATAAAGGCTTCTCAATCCGCAAGCTGGGGAGCTCGGATCCGCGCGCGTCGACCCGCGG-3'
3'-GGTAGGGCTGAGGGCCCCGGGAGCCTGTGGGGCTAGGAGGGTGTTATTTCCGAAGAGTTAGGCGTTCGACCCCTCGAGCCTAGGCGCGCGCAGCTGGGCGCC-5'


5'-AGCTCGGATCC-3'
3'-TCGAGCCTAGG-5'
      BamHI
```

Figure 7

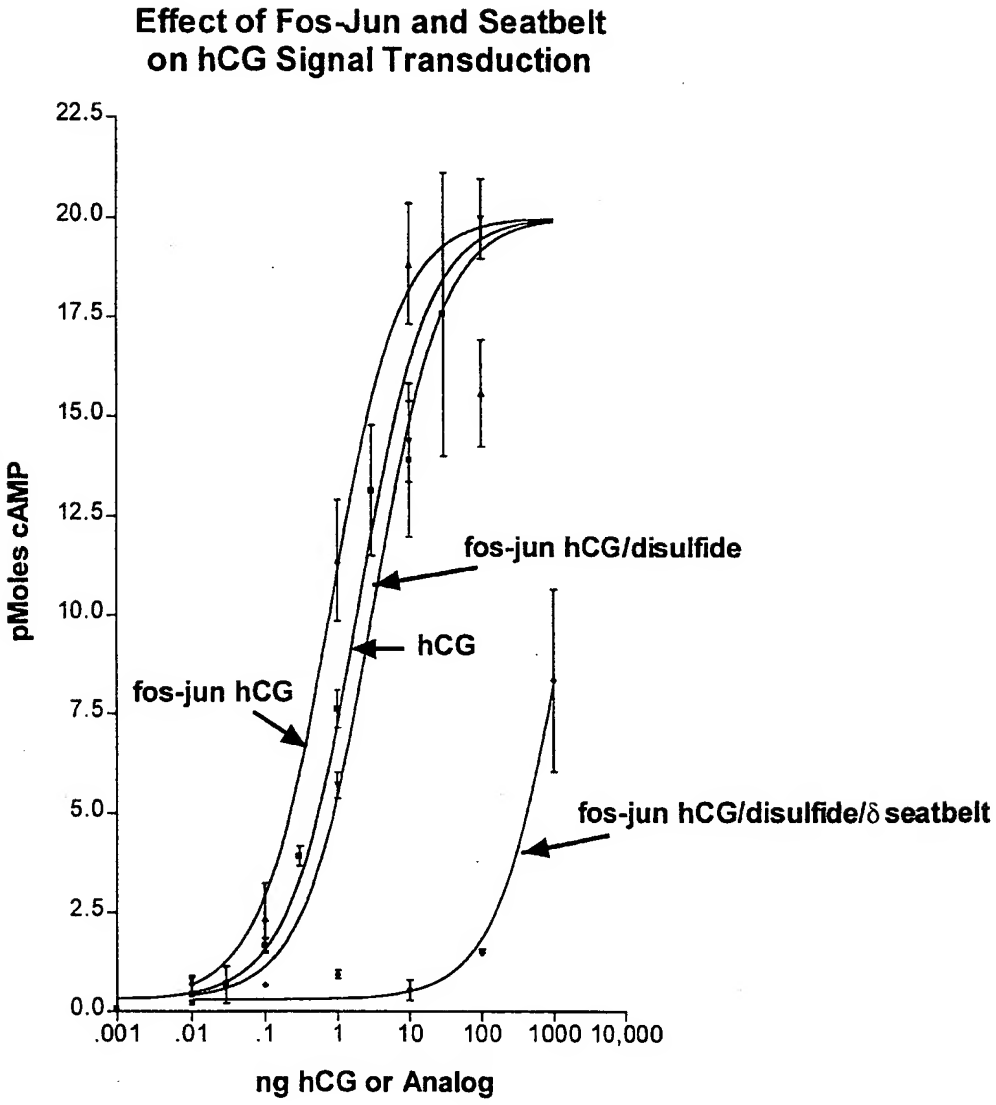
Effect of Fos-Jun and Seatbelt
on hCG Signal Transduction
pMoles cAMP
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
.001
.01
.1
1
10
100
1000
10,000
ng hCG or Analog
fos-jun hCG/disulfide
hCG
fos-jun hCG
fos-jun hCG/disulfide/δ seatbelt

Figure 8

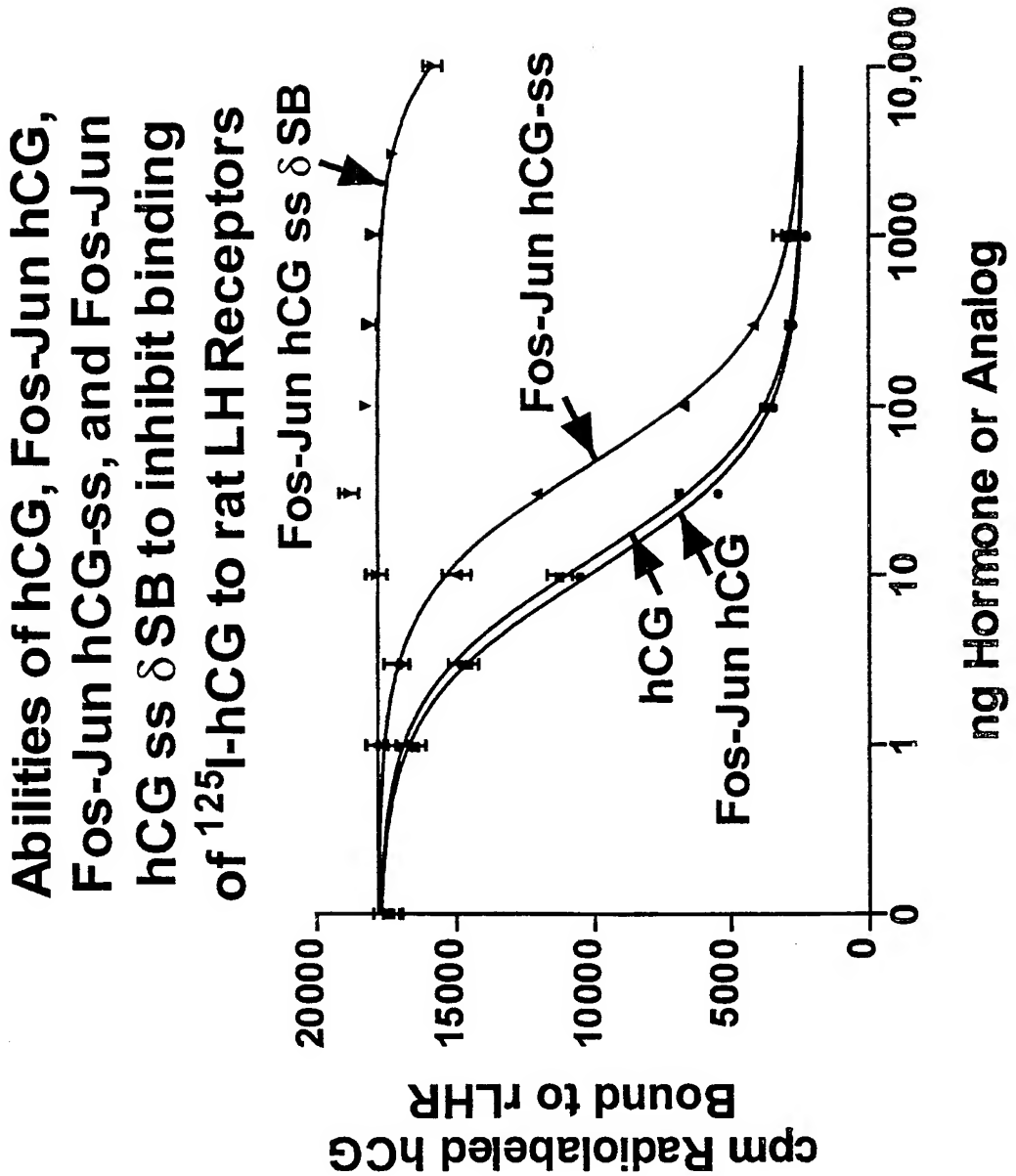
Abilities of hCG, Fos-Jun hCG, Fos-Jun hCG-ss, and Fos-Jun hCG ss δSB to inhibit binding of 125I-hCG to rat LH Receptors
Fos-Jun hCG ss δSB
Fos-Jun hCG-ss
hCG
Fos-Jun hCG
cpm Radiolabeled hCG Bound to rLHR
20000
15000
10000
5000
0
0
1
10
100
1000
10,000
ng Hormone or Analog

Figure 9

```
                                                                M  D  Y  Y  Y  R  K  Y  A  A  A  I  F
5'-CTCGAGTCTAGACCCAGCTTGGCAGTCAACCGCCCTGAACACATCCTGCAAAAAGCCCAGAGAAAGGAGCGCCATGGATTACTACAGAAAATATGCAGCTATCTTT
3'-GAGCTCAGATCTGGGTCGAACCGTCAGTTGGCGGCACTTGTGTAGGACGTTTTTCGGGTCTCTTTCCTCGCGGTACCTAATGATGTCTTTATACGTCGATAGAAA
     XhoI  XbaI                                                                                          Nhe

     L  H  V  L  H  S  A  P  D  V  Q  D  C  P  E  C  T  L  Q  E  N  P  F  F  S  Q  P  G  A  P  I  L  Q  C  M
5'-CTGCATGTTCTCCATTCCGCTCCTGATGTGCAGGATTGCCCAGAATGCACGCTACAGGAAAACCCATTCTTCTCCCAGCCCGGGTGCCCCAATACTTCAGTGCATG-
3'-GACGTACAAGAGGTAAGGCGAGGACTACACGTCCTAACGGGTCTTACGTGCGATGTCCTTTTGGGTAAGAAGAGGGTCGGCCCACGGGGTTATGAAGTCACGTAC-
                                  BsmI

     G  C  C  F  S  R  A  Y  P  T  P  L  R  S  K  K  T  M  L  V  Q  K  D  V  T  S  E  S  T  C  C  V  A  K  S
5'-GGCTGCTGCTTCTCTAGAGCATATCCCACTCCACTAAGATCTAAGAAGACTATGTTGGTCCAAAAGGACGTCACTAGTGAGTCCACTTGCTGTGTAGCTAAATCA-3'
3'-CCGACGACGAAGAGATCTCGTATAGGGTGAGGTGATTCTAGATTCTTCTGATACAACCAGGTTTCCTGCAGTGATGACTCAGGTGAACGACACATCGATTTAGT-5'
                  XbaI                BglII                                    AatII SpeI

     Y  N  R  V  T  V  M  G  G  F  K  V  E  N  H  T  A  C  H  C  S  T  C  Y  Y  H  K  S  *
5'-TATAACAGGGTCACAGTAATGGGGGGTTTCAAAGTGGAGAACCACACGGCGTGCCACTGCAGTACTTGTTATTATCACAAATCTTAAATGTTTTACCAAGTGCTG-3'
3'-ATATTGTCCCAGTGTCATTACCCCCCCAAAGTTTCACCTCTTGGTGTGCCGCACGGTGACGTCATGAACAATAATAGTGTTTAGAATTTACAAAATGGTTCACGAC-5'
                                  DraIII          PstI ScaI

5'-TCTTGATGACTGCTGATTTTCTGGAATGGAAAATTAAGTTGTTTATGTGTTTATGGCTTTGTGAGATAAAACTCTCCTTTCCTTACCATACCACTTTGACACGCT-3'
3'-AGAACTACTGACGACTAAAAGACCTTACCTTTTAATTCAACAAATCACAAATACCGAAACACTCTATTTTGAGAGGAAAAGGAATGGTATGGTGAAACTGTGCGA-5'


5'-TCAAGGATATACTGCAGCTTTACTGCCTTCCTCCTTATCCTACAGTACAATCAGCAGTCTAGTTCTTTTCATTTGGAATGAATACAGCATTAAGCTGGGGGATCC-
3'-AGTTCCTATATGACGTCGAAATGACGGAAGGAGGAATAGGATGTCATGTTAGTCGTCAGATCAAGAAAAGTAAACCTTACTTATGTCGTAATTCGACCCCCTAGG-
                                                                                                    BamHI
```

Figure 10

```
                                                     M  E  M  F  Q  G  L  L  L  L  L  L  L  S  M  G  G  T  W  A  S
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGCTGAGCATGGGCGGGACATGGGCTAGC-3'
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACGACAACGACGACGACTCGTACCCGCCCTGTACCCGATCG-5'
   XhoI  XbaI                                                                                                 NheI

   C  G  G  L  T  D  T  L  Q  A  E  T  D  Q  L  E  D  K  K  S  A  L  Q  T  E  I  A  N  L  L  K  E  K  E  K  L
5'-TGTGGTGGGTTAACCGATACCCTGCAAGCTGAAACTGATCAACTGGAAGATAAGAAATCTGCTCTGCAAACTGAAATCGCTAATCTGCTGAAAGAGAAGGAAAAGCTT-3'
3'-ACACCACCCAATTGGCTATGGGACGTTCGACTTTGACTAGTTGACCTTCTATTCTTTAGACGAGACGTTTGACTTTAGCGATTAGACGACTTTCTCTTCCTTTTCGAA-5'
                                                                                                           HindIII

   E  F  I  L  A  G  Q  D  C  P  E  C  T  L  Q  E  N  P  F  F  S  Q  P  G  A  P  I  L  Q  C  M  G  C  C  F  S
5'-GAGTTCATCCTGGCCGGCCAAGATTGTCCGGAATGCACGCTACAGGAAAACCCATTCTTCTCCCAGCCGGGTGCCCCAATACTTCAGTGCATGGGCTGCTGCTTCTCT-3'
3'-CTCAAGTAGGACCGGCCGGTTCTAACAGGCCTTACGTGCGATGTCCTTTTGGGTAAGAAGAGGGTCGGCCCACGGGGTTATGAAGTCACGTACCCGACGACGAAGAGA-5'
               NgoMI         BspEI BsmI

   R  A  Y  P  T  P  L  R  S  K  K  T  M  L  V  Q  K  D  V  T  S  E  S  T  C  C  V  A  K  S  Y  N  R  V  T  V
5'-AGAGCATATCCCACTCCACTAAGATCTAAGAAGACTATGTTGGTCCAAAAGGACGTCACTAGTGAGTCCACTTGCTGTGTAGCTAAATCATATAACAGGGTCACAGTA-3'
3'-TCTCGTATAGGGTGAGGTGATTCTAGATTCTTCTGATACAACCAGGTTTTCCTGCAGTGATGACTCAGGTGAACGACACATCGATTTAGTATATTGTCCCAGTGTCAT-5'
   XbaI                  BglII                                AatII SpeI

   M  G  G  F  K  V  E  N  H  T  A  C  H  C  S  T  C  Y  Y  H  K  S  *
5'-ATGGGGGGTTTCAAAGTGGAGAACCACACGGCGTGCCACTGCAGTACTTGTTATTATCACAAATCTTAAATGTTTTACCAAGTGCTGTCTTGATGACTGCTGATTTTC-3'
3'-TACCCCCCAAAGTTTCACCTCTTGGTGTGCCGCACGGTGACGTCATGAACAATAATAGTGTTTAGAATTTACAAAATGGTTCACGACAGAACTACTGACGACTAAAAG-5'
                           DraIII       PstI ScaI

5'-TGGAATGGAAAATTAAGTTGTTTAGTGTTTATGGCTTTGTGAGATAAAACTCTCCTTTTCCTTACCATACCACTTTGACACGCTTCAAGGATATACTGCAGCTTTACT-3'
3'-ACCTTACCTTTTAATTCAACAAATCACAAATACCGAAACACTCTATTTTGAGAGGAAAAGGAATGGTATGGTGAAACTGTGCGAAGTTCCTATATGACGTCGAAATGA-5'

5'-GCCTTCCTCCTTATCCTACAGTACAATCAGCAGTCTAGTTCTTTTCATTTGGAATGAATACAGCATTAAGCTGGGGGATCC-3'
3'-CGGAAGGAGGAATAGGATGTCATGTTAGTCGTCAGATCAAGAAAAGTAAACCTTACTTATGTCGTAATTCGACCCCCTAGG-5'
                                                                              BamHI
```

Figure 10A
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGLTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAG-KSKR-
QDCPECTLQENPFFSQPGAPILQCMGCC-
FSRAYPTPLRSKKTMLVQKNVTSESTCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHKS-Carboxyl

Figure 10B
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMG-KSKR-
LRPRCRPINATLAVEKEGCPVCITVNTT-

ICAGYCPTMTRVLQGVLPALPQVVCNYRDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCD
DPRFQDSSSSKAPPPSLPSPSRLPG-
PSDTPILPQ-Carboxyl

Figure 11

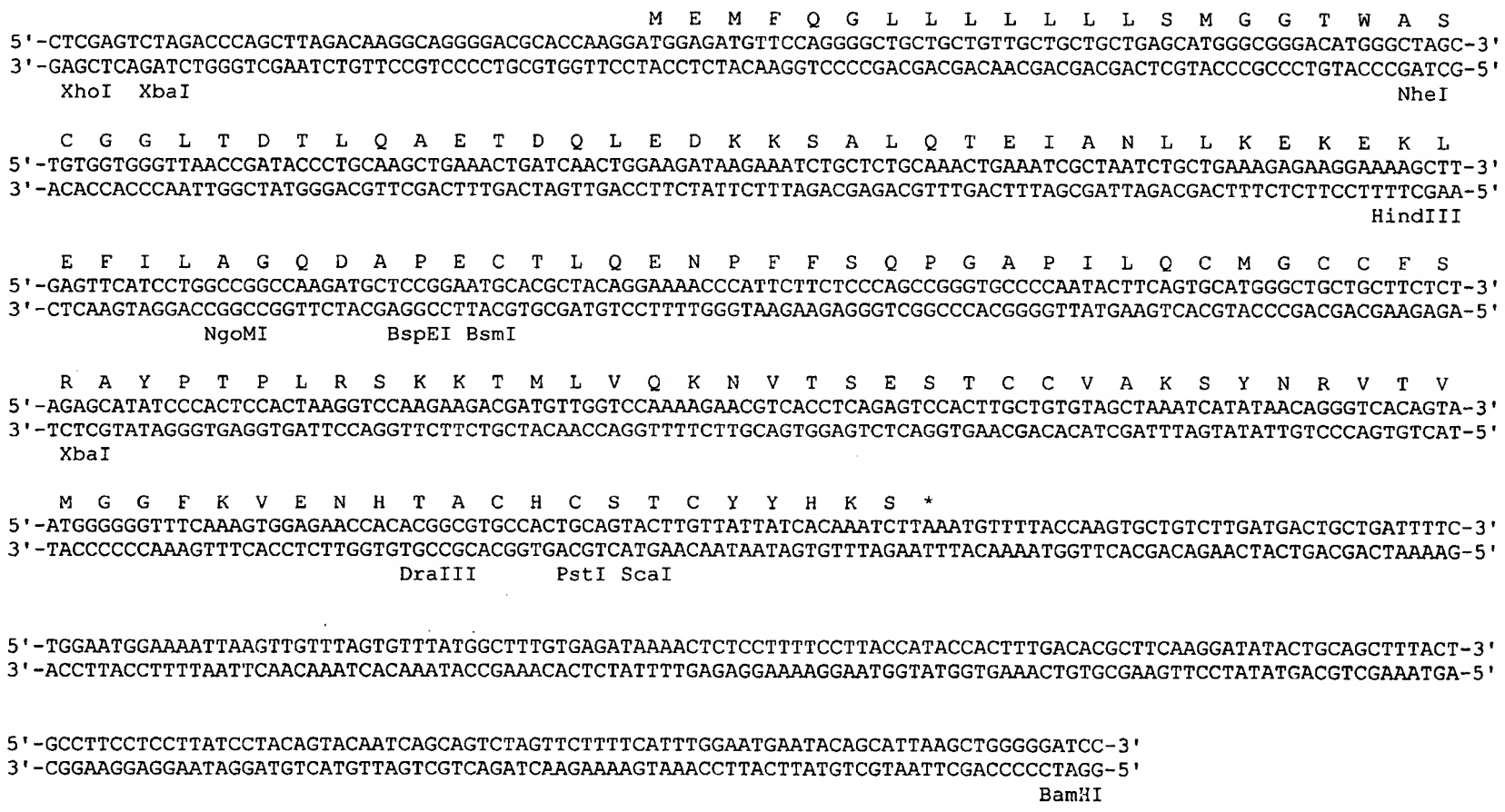

Figure 12

```
                                                M  E  M  F  Q  G  L  L  L  L  L  L  S  M  G  G  T  W
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGAGCATGGGCGGGACATGG-3'
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACGACAACGACTCGTACCCGCCCTGTACC-5'
      XhoI  XbaI

     A  S  C  G  G  G  R  I  A  R  L  E  E  K  V  K  T  L  K  A  Q  N  S  E  L  A  S  T  A  N  M  L  R  E  Q
5'-GCTAGCTGTGGCGGCCGCATTGCTAGATTGGAAGAGAAAGTTAAAACTCTGAAGGCCCAAAACAGCGAACTGGCTTCCACTGCTAATATGCTGCGTGAACAA-3'
3'-CGATCGACACCGCCGGCGTAACGATCTAACCTTCTCTTTCAATTTTGAGACTTCCGGGTTTTGTCGCTTGACCGAAGGTGACGATTATACGACGCACTTGTT-5'
      NheI

     V  A  Q  L  K  Q  K  V  M  G  L  R  P  R  C  R  P  I  N  A  T  L  A  V  E  K  E  G  C  P  V  C  I  T
5'-GTCGCTCAACTGAAGCAAAAGGTTATGGGTTTGCGCCCTAGGTGCCGCCCCATCAATGCCACCCTGGCTGTGGAGAAGGAGGGCTGCCCCGTGTGCATCACC-3'
3'-CAGCGAGTTGACTTCGTTTTCCAATACCCAAACGCGGGATCCACGGCGGGTAGTTACGGTGGGACCGACACCTCTTCCTCCCGACGGGGCACACGTAGTGG-5'
                                AvrII BanI

     V  N  T  T  I  C  A  G  Y  C  P  T  M  T  R  V  L  Q  G  V  L  P  A  L  P  Q  V  V  C  N  Y  R  D  V
5'-GTCAACACCACCATCTGTGCCGGCTGTTGTCCTACCATGACACGTGTGCTGCAGGGCGTCCTCCCGGCCCTGCCTCAGGTGGTGTGCAACTACCGCGATGTG-3'
3'-CAGTTGTGGTGGTAGACACGGCCGACAACAGGATGGTACTGTGCACACGACGTCCCGCAGGAGGGCCGGGACGGAGTCCACCACACGTTGATGGCGCTACAC-5'
                                      PstI
                                    NgoMI

     R  F  E  S  I  R  L  P  G  C  P  R  G  V  N  P  V  V  S  Y  A  V  A  L  S  C  Q  C  A  L  C  R  R  S
5'-CGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCGCGGCGTGAACCCCGTGGTCTCCTACGCCGTGGCTCTCAGCTGTCAATGTGCACTCTGCCGCCGCAGC-3'
3'-GCGAAGCTCAGGTAGGCCGAGGGACCGACGGGCGCCGCCGCACTTGGGGCACCAGAGGATGCGGCACCGAGAGTCGACAGTTACACGTGAGACGGCGGCGTCG-5'

   T  T  D  C  G  G  P  K  D  H  P  L  T  C  D  D  P  R  F  Q  D  S  S  S  S  K  A  P  P  P  S  L  P  S
5'-ACCACTGACTGCGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAGGACTCCTCTTCCTCAAAGGCCCCTCCCCCCAGCCTTCCAAGC-3'
3'-TGGTGACTGACGCCCCCAGGGTTCCTGGTGGGGAACTGGACACTACTGGGGGCGAAGGTCCTGAGGAGAAGGAGTTTCCGGGGAGGGGGGTCGGAAGGTTCG-5'

   P  S  R  L  P  G  P  S  D  T  P  I  L  P  Q  *
5'-CCATCCCGACTCCCGGGCCCTCGGACACCCCGATCCTCCCACAATAAAGGCTTCTCAATCCGCAAGCTGGGGAGCTCGGATCCGCGCGCTCGACCCGCGG-3'
3'-GGTAGGGCTGAGGGCCCCGGAGCCTGTGGGCTAGGAGGGTGTTATTTCCGAAGAGTTAGGCGTTCGACCCCTCGAGCCTAGGCGCGCGCAGCTGGGCGCC-5'


5'-AGCTCGGATCC-3'
3'-TCGAGCCTAGG-5'
      BamHI
```

Figure 13

```
                                    M  E  M  F  Q  G  G  L  L  L  L  L  L  L  S  M  G  G  T  W
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGAGCATGGGCGGGACATGG-3'
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACGACAACGACGACTCGTACCCGCCCTGTACC-5'
      XhoI  XbaI

     A  S  C  G  G  R  I  A  R  L  E  E  K  V  K  T  L  K  A  Q  N  S  E  L  A  S  T  A  N  M  L  R  E  Q
5'-GCTAGCTGTGGCGGCCGCATTGCTAGATTGGAAGAGAAAGTTAAAACTCTGAAGGCCCAAAACAGCGAACTGGCTTCCACTGCTAATATGCTGCGTGAACAA-3'
3'-CGATCGACACCGCCGGCGTAACGATCTAACCTTCTCTTTCAATTTTGAGACTTCCGGGTTTTGTCGCTTGACCGAAGGTGACGATTATACGACGCACTTGTT-5'
       NheI

     V  A  Q  L  K  Q  K  V  M  G  L  R  P  R  C  R  P  I  N  A  T  L  A  V  E  K  E  G  C  P  V  A  I  T
5'-GTCGCTCAACTGAAGCAAAAGGTTATGGGTTTGCGCCCTAGGTGTCGTCCTATTAATGCTACTCTGGCTGTTGAGAAGGAAGGTTGTCCTGTGGCCATTACT-3'
3'-CAGCGAGTTGACTTCGTTTTCCAATACCCAAACGCGGGATCCACAGCAGGATAATTACGATGAGACCGACAACTCTTCCTTCCAACAGGACACCGGTAATGA-5'
                                  AvrII BanI

     V  N  N  T  T  I  C  A  G  Y  C  P  T  M  T  R  V  L  Q  G  V  L  P  A  L  P  Q  V  V  C  N  Y  R  D  V
5'-GTTAACACTACCATCTGTGCCGGCTGTTGTCCTACCATGACACGTGTGCTGCAGGGCGTCCTCCCGGCCCTGCCTCAGGTGGTGTGTGCAACTACCGCGATGTG-3'
3'-CAATTGTGATGGTAGACACGGCCGACAACAGGATGGTACTGTGCACACGACGTCCCGCAGGAGGGCCGGGACGGAGTCCACCACACGTTGATGGCGCTACAC-5'
                                      NgoMI
                                                PstI

     R  F  E  S  I  R  L  P  G  C  P  R  G  V  N  P  V  V  S  Y  A  V  A  L  S  C  Q  C  A  L  C  R  R  S
5'-CGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCGCGGCGTGAACCCCGTGGTCTCCTACGCCGTGGCTCTCAGCTGTCAATGTGCCACTCTGCCGCCGCAGC-3'
3'-GCGAAGCTCAGGTAGGCCGAGGGACCGACGGGCGCGCCGCACTTGGGGCACCAGAGGATGCGGCACCGAGAGTCGACAGTTACACGTGAGACGGCGGCGTCG-5'

     T  T  D  C  G  G  G  P  K  D  H  P  L  T  C  D  D  P  R  F  Q  D  S  S  S  S  K  A  P  P  P  S  L  P  S
5'-ACCACTGACTGCGGGGGGTCCCAAGGACCACCCCTTGACCTGTGATGACCCCCGCTTCCAGGACTCCTCTTCCTCAAAGGCCCCTCCCCCCAGCCTTCCAAGC-3'
3'-TGGTGACTGACGCCCCCCAGGGTTCCTGGTGGGGAACTGGACACTACTGGGGGCGAAGGTCCTGAGGAGAAGGAGTTTCCGGGGAGGGGGGTCGGAAGGTTCG-5'

     P  S  R  L  P  G  P  S  D  T  P  I  L  P  Q  *
5'-CCATCCCGACTCCCGGGGCCCTCGGACACCCCGATCCTCCCACAATAAAGGCTTCTCAATCCGCAAGCTGGGAGCTCGGATCCGCGCGGCGTCGACCCGCGG-3'
3'-GGTAGGGCTGAGGGCCCCGGGAGCCTGTGGGGCTAGGAGGGTGTTATTTCCGAAGAGTTAGGCGTTCGACCCCTCGAGCCTAGGCGCGCCGCAGCTGGGCGCC-5'


5'-AGCTCGGATCC-3'
3'-TCGAGCCTAGG-5'
      BamHI
```

Figure 14

```
                                    M  E  M  F  Q  G  L  L  L  L  L  L  L  S  M  G  G  T  W
5'-CTCGAGTCTAGACCCAGCTTAGACAAGGCAGGGGACGCACCAAGGATGGAGATGTTCCAGGGGCTGCTGCTGTTGCTGCTGAGCATGGGCGGGACATGG-3'
3'-GAGCTCAGATCTGGGTCGAATCTGTTCCGTCCCCTGCGTGGTTCCTACCTCTACAAGGTCCCCGACGACACAACGACGACTCGTACCCGCCCTGTACC-5'
      XhoI  XbaI

    A  S  C  G  G  R  I  A  R  L  E  E  K  V  K  T  L  K  A  Q  N  S  E  L  A  S  T  A  N  M  L  R  E  Q
5'-GCTAGCTGTGGCGGCCGCATTGCTAGATTGGAAGAGAAAGTTAAAACTCTGAAGGCCCAAACAGCGAACTGGCTTCCACTGCTAATATGCTGCGTGAACAA-3'
3'-CGATCGACACCGCCGGCGTAACGATCTAACCTTCTCTTTCAATTTTGAGACTTCCGGGTTTTGTCGCTTGACCGAAGGTGACGATTATACGACGCACTTGTT-5'
     NheI

    V  A  Q  L  K  Q  K  V  M  G  L  L  R  P  R  C  R  P  I  N  A  T  L  A  V  E  K  E  G  C  P  V  A  I  T
5'-GTCGCTCAACTGAAGCAAAAGGTTATGGGTTTGCGCCCTAGGTGTCGTCCTATTAATGCTACTCTGGCTGTTGAGAAGGAAGGTTGTCCTGTGGCCATTACT-3'
3'-CAGCGAGTTGACTTCGTTTTTCCAATACCCAAACGCGGGATCCACAGCAGGATAATTACGATGAGACCGACAACTCTTCCTTCCAACAGGACACCGGTAATGA-5'
                                  AvrII BanI

    V  N  T  T  I  C  A  G  Y  C  P  T  M  T  R  V  L  Q  G  V  L  P  A  L  P  Q  V  V  C  N  Y  R  D  V
5'-GTTAACACTACCATCTGTGCCGGCTGTTGTCCTACCATGACACGTGTGCTGCAGGGCGTCCTCCCGGCCCTGCCTCAGGTGGTGTGCAACTACCGCGATGTG-3'
3'-CAATTGTGATGGTAGACACGGCCGACAACAGGATGGTACTGTGCACACGACGTCCCGCAGGAGGGCCGGGACGGAGTCCACCACACGTTGATGGCGCTACAC-5'
                                  PstI
                    NgoMI

    R  F  E  S  I  R  L  P  G  C  P  R  G  V  N  P  V  V  S  Y  A  V  A  L  S  C  Q  C  A  L  *
5'-CGCTTCGAGTCCATCCGGCTCCCTGGCTGCCCGCGCGGCGTGAACCCCGTGGTCTCCTACGCCGTGGCTCTCAGCTGTCAATGCGCCGCTTTAAAGGATCC-3'
3'-GCGAAGCTCAGGTAGGCCGAGGGACCGACGGGCGCGCCGCACTTGGGGCACCAGAGGATGCGGCACCGAGAGTCGACAGTTACGCGCGAAATTTCCTAGG-5'
                                                  BsshII DraI BamHI
```

Figure 15

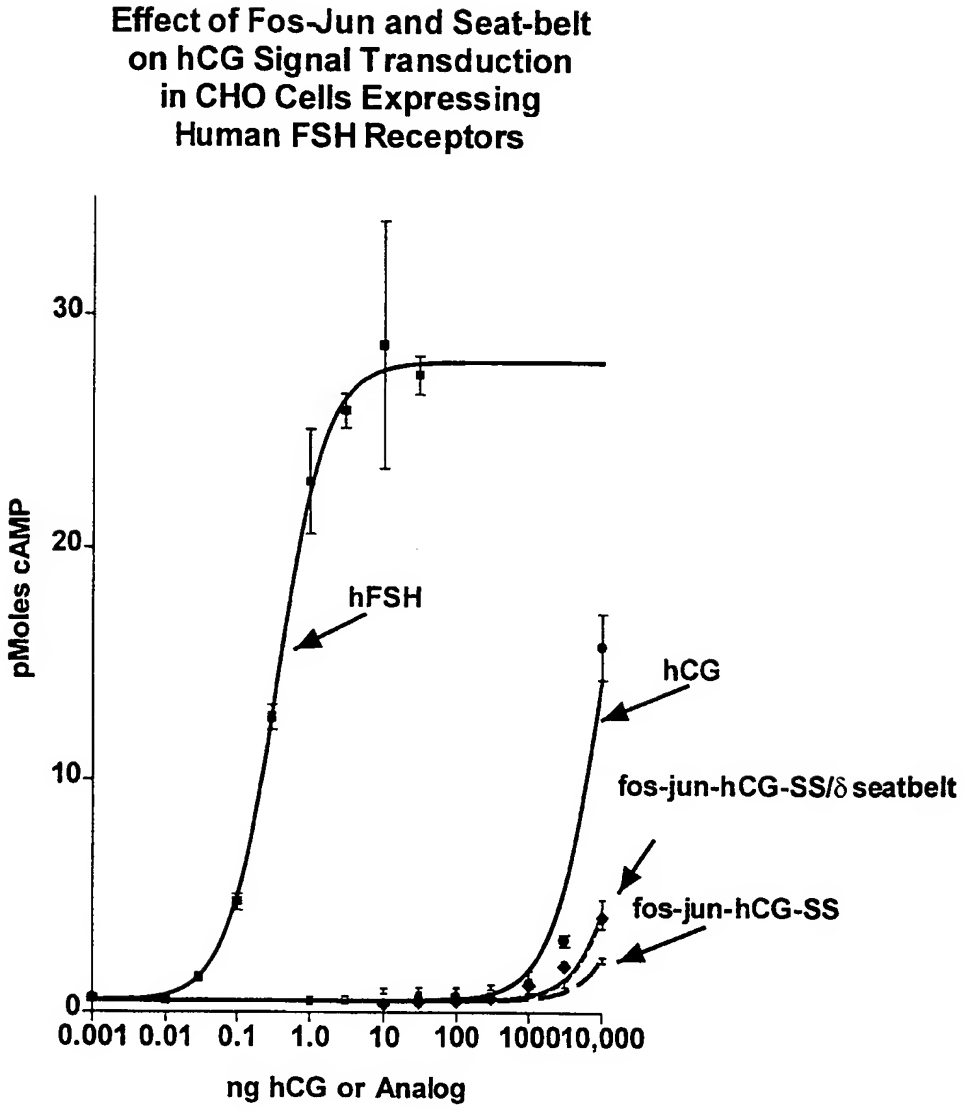
Effect of Fos-Jun and Seat-belt
on hCG Signal Transduction
in CHO Cells Expressing
Human FSH Receptors
pMoles cAMP
30
20
10
0
hFSH
hCG
fos-jun-hCG-SS/δ seatbelt
fos-jun-hCG-SS
0.001 0.01 0.1 1.0 10 100 100010,000
ng hCG or Analog

Figure 16

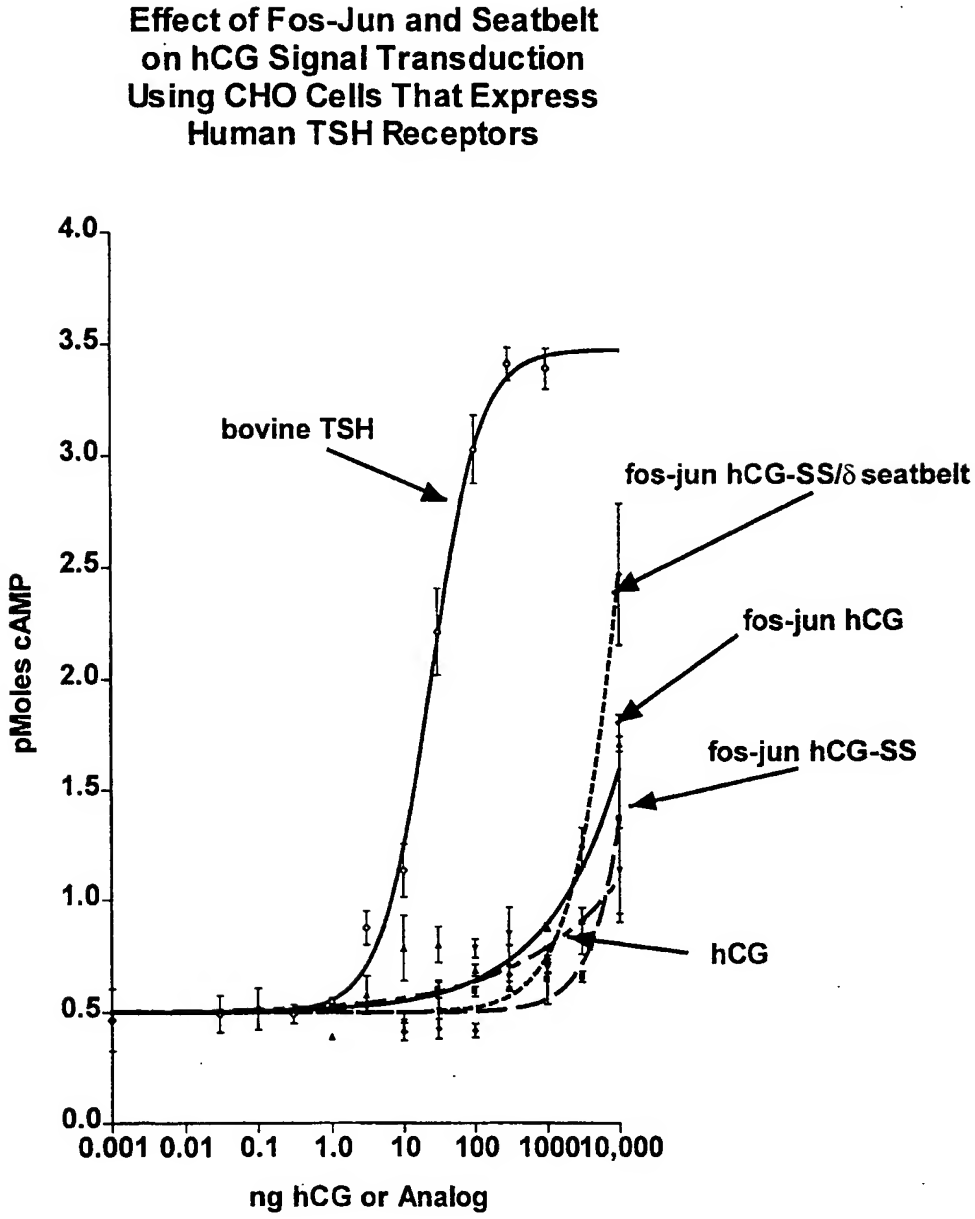

Figure 17

Full-length hCG α-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGLTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAG-KSKR-APDVQDCPECTLQENPFFSQPGAPILQC-
MGCCFSRAYPTPLRSKKTMLVQKNVTSESTCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHKS-Carboxyl

Full-length hCG β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMG-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCIT-
VNTTICAGYCPTMTRVLQGVLPALPQVVCNYRDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPPSLPSPS-
RLPGPSDTPILPQ-Carboxyl

Full-length hLH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMG-KSKR-SREPLRPWCHPINAILAVEKEGCPVCIT-
VNTTICAGYCPTMMRVLQAVLPPLPQVVCTYRDVRFESIRLPGCPRGVDPVVSFPVALSCRCALCRRSTSDCGGPKDHPLTCDHPQ-Carboxyl

Full-length hFSH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMGSKEPLR-KSKR-NSCELTNITIAIEKEECRFCIS-
INTTWCAGYCYTRDLVYKDPARPKIQKTCTFKELVYETVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE-carboxyl

Full-length hTSH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMGSKEPLRG-KSKR-FCIPTEYTMHIERRECAYCLT-
INTTICAGYCMTRDINGKLFLPKYALSQDVCTYRDFIYRTVEIPGCPLHVAPYFSYPVALSCKCGKCNTDYSDCIHEAIKTNYCTKPQKSY-carboxyl

Full-length hCG/hFSH β-subunit chimera:
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMG-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCIT-
VNTTICAGYCPTMTRVLQGVLPALPQVVCNYRDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCDSDSTDCTVRGLGPSYCSFGEMKESSSSKAPPPSLPSPS-
RLPGPSDTPILPQ-Carboxyl

Full-length hCG/hTSH β-subunit chimera:
Amino-MEMFQGLLLLLLLSMGGTWA-SCGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMG-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCIT-
VNTTICAGYCPTMTRVLQGVLPALPQVVCNYRDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCNTDYSDCIHEAIKTNYCTKPQKSYSSSSKAPPPSLPSPS-
RLPGPSDTPILPQ-Carboxyl

Figure 18

Full-length hCG α-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKD-
STYSMSSTLTWTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC]G-KSKR-APDVQDCPECTLQENPFFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQKNVTSE-
STCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHKS-Carboxyl

Full-length hCG β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDC]G-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ-Carboxyl

Full-length hLH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDC]G-KSKR-SREPLRPWCHPINAILAVEKEGCPVCITVNTTICAGYCPTMMRVLQAVLPPLPQVVCTY-
RDVRFESIRLPGCPRGVDPVVSFPVALSCRCALCRRSTSDCGGPKDHPLTCDHPQ-Carboxyl

Full-length hFSH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDC]G-KSKR-NSCELTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDPARPKIQKTCTFKELVYE-
TVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE-carboxyl

Full-length hTSH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDC]G-KSKR-FCIPTEYTMHIERRECAYCLTINTTICAGYCMTRDINGKLFLPKYALSQDVCTYRDFIY-
RTVEIPGCPLHVAPYFSYPVALSCKCGKCNTDYSDCIHEAIKTNYCTKPQKSY-carboxyl

Full-length hCG/hFSH β-subunit chimera:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDC]G-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCDSDSTDCTVRGLGPSYCSFGEMKESSSSKAPPPSLPSPSRLPGPSDTPILPQ-Carboxyl

Full-length hCG/hTSH β-subunit chimera:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDC]G-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCNTDYSDCIHEAIKTNYCTKPQKSYSSSSSKAPPPSLPSPSRLPGPSDTPILPQ-Carboxyl

Figure 19

Full-length hCG α-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKD-
STYSMSSTLTWTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEA]G-KSKR-APDVQDCPECTLQENPFFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQKNVTSE-
STCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHKS-Carboxyl

Full-length hCG β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDA]G-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ-Carboxyl

Full-length hLH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDA]G-KSKR-SREPLRPWCHPINAILAVEKEGCPVCITVNTTICAGYCPTMMRVLQAVLPPLPQVVCTY-
RDVRFESIRLPGCPRGVDPVVSFPVALSCRCALCRRSTSDCGGPKDHPLTCDHPQ-Carboxyl

Full-length hFSH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDA]G-KSKR-NSCELTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDPARPKIQKTCTFKELVYE-
TVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE-carboxyl

Full-length hTSH β-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDA]G-KSKR-FCIPTEYTMHIERRECAYCLTINTTICAGYCMTRDINGKLFLPKYALSQDVCTYRDFIY-
RTVEIPGCPLHVAPYFSYPVALSCKCGKCNTDYSDCIHEAIKTNYCTKPQKSY-carboxyl

Full-length hCG/hFSH β-subunit chimera:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDA]G-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCDSDSTDCTVRGLGPSYCSFGEMKESSSSKAPPPSLPSPSRLPGPSDTPILPQ-Carboxyl

Full-length hCG/hTSH β-subunit chimera:
Amino-MEMFQGLLLLLLLSMGGTWA-[variable region]-[SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLESDL-
YTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDA]G-KSKR-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCNTDYSDCIHEAIKTNYCTKPQKSYSSSSKAPPPSLPSPSRLPGPSDTPILPQ-Carboxyl

Figure 20

Full-length hCG β-subunit containing a Jun dimerization domain at its C-terminus:
Amino-MEMFQGLLLLLLLSMGGTWA-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPSLPSPSRLPGPSDTPILPQ-
RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVM-Carboxyl

Full-length hCG β-subunit containing a linker and a Jun dimerization domain at its C-terminus:
Amino-MEMFQGLLLLLLLSMGGTWA-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPSLPSPSRLPGPSDTPILPQ-
GSGSGS-RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVM-Carboxyl

hLH β-subunit containing hCG C-terminus, a linker, and a Jun dimerization domain at its C-terminus:
Amino-MEMFQGLLLLLLLSMGGTWA-SREPLRPWCHPINAILAVEKEGCPVCITVNTTICAGYCPTMMRVLQAVLPPLPQVVCTY-
RDVRFESIRLPGCPRGVDPVVSFPVALSCRCALCRRSTSDCGGPKDHPLTCDDPRFQDSSSSKAPPSLPSPSRLPGPSDTPILPQ-
GSGSGS-RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVM-Carboxyl

hFSH β-subunit containing hCG C-terminus, a linker, and a Jun dimerization domain at its C-terminus:
Amino-MEMFQGLLLLLLLSMGGTWA-NSCELTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDPARPKIQKTCTFKELVYE-
TVRVPGCAHHADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKESSSSKAPPPSLPSPSRLPGPSDTPILPQ-
GSGSGS-RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVM-Carboxyl

hTSH β-subunit containing hCG C-terminus, a linker, and a Jun dimerization domain at its C-terminus:
Amino-MEMFQGLLLLLLLSMGGTWA-FCIPTEYTMHIERRECAYCLTINTTICAGYCMTRDINGKLFLPKYALSQDVCTYRDFIY-
RTVEIPGCPLHVAPYFSYPVALSCKCGKCNTDYSDCIHEAIKTNYCTKPQKSYSSSSKAPPPSLPSPSRLPGPSDTPILPQ-
GSGSGS-RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVM-Carboxyl

Full-length hCG β-subunit containing a Jun dimerization domain at its C-terminus expected to form an intersubunit disulfide with the α-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPSLPSPSRLPGPSDTPILPQ-
CGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVM-Carboxyl

Full-length hCG β-subunit containing a linker and a Jun dimerization domain at its C-terminus expected to form an intersubunit disulfide with the α-subunit:
Amino-MEMFQGLLLLLLLSMGGTWA-SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNY-
RDVRFESIRLPGCPRGVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPSLPSPSRLPGPSDTPILPQ-
GSGSGS-CGGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVM-Carboxyl

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08018

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12N 15/16; C07K 14/48, 14/49, 14/495, 14/51, 14/59
US CL :435/69.4, 69.7; 530/397, 398, 399
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.4, 69.7; 530/397, 398, 399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,567,584 A (SLEDZIEWSKI et al) 22 October 1996, col. 3, line 14 to col. 7, line 63.	1-19
A	KELLENBERGER et al. Serine protease inhibition by insect peptides containing a cysteine knot and a triple-stranded β-sheet. J. Biol. Chem. 27 October 1995, Vol. 270, No. 43, pages 25514-25519.	1-19
A	PALLAGHY et al. A common structural motif incorporating a cystine knot and a triple-stranded β-sheet in toxic and inhibitory polypeptides. Protein Science. October 1994, Vol. 3, pages 1833-1839.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
30 JUNE 1999	18 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHRISTINE SAOUD Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08018

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOLLAND et al. Nerve growth factor in different crystal forms displays structural flexibility and reveals zinc binding sites. June 1994, Vol. 239, pages 385-400.	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08018

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, MEDLINE, EMBASE, BIOSIS
search terms: cysteine knot, dimerization domain, dimerization, glycoprotein, FSH, LH, TSH, HCG, fusion, chimer?, subunit, activin, PDGF